Supplementary Materials

1. General materials and methods

Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). Recombinant plasmid DNA was purified with a Qiaprep Kit from Qiagen. Gel extraction of DNA fragments and restriction endonuclease clean up were performed using an Illustra GFX PCR DNA and Gel Band Purification Kit from GE Healthcare. DNA sequencing was performed by Genewiz (Boston, MA). Nickel-nitrilotriacetic acid-agarose (Ni-NTA) resin was purchased from Qiagen and Thermo Scientific. SDS-PAGE gels were purchased from BioRad. Protein concentrations were determined by absorption at 280 nm using ExPASy ProtParam (http://web.expasy.org/protparam/) to calculate the extinction coefficients. Optical densities of *E. coli* cultures were determined with a DU 730 Life Sciences UV/Vis spectrophotometer (Beckman Coulter) by measuring absorbance at 600 nm. Analytical HPLC was performed on a Dionex Ultimate 3000 instrument (Thermo Scientific).

Analysis of cremeomycin, 2-hydroxy-4-methoxybenzoic acid (2,4-HMBA), 3-amino-4-hydroxybenzoic acid (3,4-AHBA) and 3-acetamido-4-hydroxybenzoic acid (3,4-NAcHBA) by LC/MS as well as analysis of cremeomycin and 2,4-HMBA by LC/MS/MS were carried out on an Agilent 1290 Infinity UHPLC system (Agilent Technologies, Palo Alto, CA) coupled to a maXis impact UHR time-of-flight mass spectrometer system (Bruker Daltonics Inc) equipped with an electrospray ionization (ESI) source. Data were acquired with Bruker Daltonics HyStar software version 3.2 for UHPLC and Compass OtofControl software version 3.4 for mass spectrometry, and they were processed with Bruker Compass DataAnalysis software version 4.2.

For the UHPLC analysis of cremeomycin, 2,4-HBMA, and 3,4-NAcHBA, 8 µL samples were injected onto the UHPLC including a G4220A binary pump with a built-in vacuum degasser and a thermostatted G4226A high performance autosampler. An XTerra MS C18 analytical column (2.1x100 mm, 3.5 µm) from Waters Corporation was used at the flow rate of 0.3 mL/min using 0.2% acetic acid in water as mobile phase A and 0.2% acetic acid in acetonitrile as mobile phase B. The column temperature was maintained at

room temperature. The following gradient was applied: 0-3 min: 5% B isocratic, 3-12 min: 5-100% B, 12-14 min: 100% B isocratic, 14-14.5 min: 100-5%B, 14.5-20.5 min: 5% B isocratic.

For the UHPLC analysis of 3,4-AHBA, 1.5 µL samples were injected. A Cogent Diamond Hydride analytical HPLC column (2.1x150 mm, 4 µm) from MicroSolv Technology Corporation was used at the flow rate of 0.4 mL/min using 0.2% acetic acid in water as mobile phase A and 0.2% acetic acid in acetonitrile as mobile phase B. The column temperature was maintained at room temperature. The following gradient was applied: 0-5 min: 100-75% B, 5-6 min: 75-60% B, 6-7 min: 60% B isocratic, 7-9 min: 60-50% B, 9-15 min: 50% B isocratic, 15-16 min: 50-100% B, 16-21 min: 100% B isocratic.

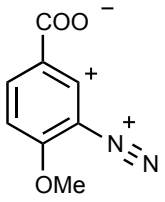
For the UHPLC system for FAD and FMN, 1 µL sample was injected. The same XTerra MS C18 analytical column (2.1x100 mm, 3.5 µm) used for detection of cremeomycin on qTOF was used on Agilent QQQ at the flow rate of 0.3 mL/min using the same mobile phase for detection of cremeomycin on QQQ. The column temperature was maintained at room temperature. The following gradient was applied: 0-2 min: 0% B isocratic, 2-5 min: 0-52% B, 5-5.1 min: 52-100% B, 5.1-5.2 min: 100-0%B isocratic, 5.2-7 min: 0% B isocratic.

For the MS portion of the LC/MS, the ESI mass spectra data were recorded on a positive ionization mode for cremeomycin and on a negative mode for 2,4-HMBA, 3,4-NAcHBA, and 3,4-AHBA, all with a mass range of m/z 50 to 1200; calibration mode, HPC; spectra rate, 1.00 Hz; capillary voltage, 3800 V for ESI+ & 3400 V for ESI-; nebulizer pressure, 25.0 psi for cremeomycin, 2,4-HMBA, and 3,4-NAcHBA, and 35.0 psi for 3,4-AHBA; drying gas (N_2) flow, 9.3 L/min for cremeomycin, 2,4-HMBA and 3,4-NAcHBA, and 10.7 L/min for 3,4-AHBA; temperature, 220 ºC for all. A mass window of ± 0.005 Da was used to extract the ion of $[M+Na]^+$ for cremeomycin & $[M-H]^-$ for 2,4-HMBA, 3,4-NAcHBA, and 3,4-AHBA. Targets were considered detected when the mass accuracy was less than 5 ppm and there was a match of isotopic pattern between the observed and

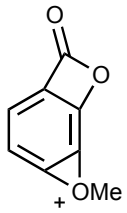
the theoretical ones plus a match of retention time between those in real samples and standards.

For the MS portion of the LC/MS/MS, the ESI mass spectra data were recorded on a positive ionization mode for cremeomycin and negative mode for 2,4-HMBA; calibration mode, HPC; spectra rate, 1.00 Hz; capillary voltage, 3800 V for ESI+ & 3400 V for ESI-; nebulizer pressure, 25.0 psi; drying gas flow, 9.3 L/min; temperature, 220 ºC. The precursors used for MS/MS of cremeomycin and 2,4-HMBA were m/z 195.0400 ($[M+H]^+$, collision energy (CE) = 15.0 eV) and 167.0350 ($[M-H]^-$, CE = 35.0 eV) respectively, both with the isolation width of 4.00 m/z. Nitrogen was used as the drying and collision gas.

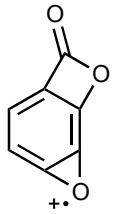
A mass window of ± 0.005 Da was used to extract the fragment ions $[C_7H_2O_3]^{+\cdot}$ (fragment ion c) for detection of cremeomycin and $[C_7H_7O_2]^-$ (fragment ion e) or $[C_6H_4O_2]^{-\cdot}$ (fragment ion f) for detection of 2,4-HMBA. Targets were considered detected when the mass accuracy was less than 5 ppm for the precursors and there was a match of MS/MS fragmentation patterns between the sample and the standards of cremeomycin (fragment ions a – d) and 2,4-HBMA (fragment ions e – g) plus a match of retention time between the sample and the standard. The following are the proposed structures of fragment ions.



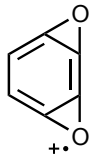
Fragment ion **a**



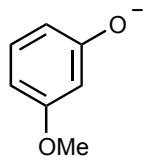
Fragment ion **b**



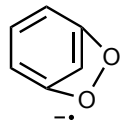
Fragment ion **c**



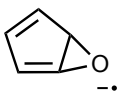
Fragment ion **d**



Fragment ion **e**



Fragment ion **f**



Fragment ion **g**

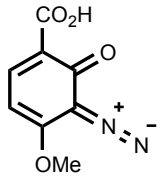
Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on Varian Inova-500 (500 MHz, 125 MHz) NMR spectrometers. Chemical shifts are reported in parts per million downfield from tetramethylsilane using the solvent resonance as internal standard for ^{1}H (DMSO-d_6 = 2.50 ppm, $CDCl_3$ = 7.26 ppm) and ^{13}C (DMSO-d_6= 39.52 ppm, $CDCl_3$ = 77.25 ppm). Data are reported as follows: chemical shift, integration multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, q=quartet, qt=quintet), coupling constant, integration, and assignment. All chemicals were obtained from Sigma-Aldrich except where noted. All isotopically labeled compounds were obtained from Cambridge Isotope Laboratories. Solvents were obtained from Sigma-Aldrich except hexanes (Macron Fine Chemicals), ethyl acetate and isopropanol (VWR), methanol and diethyl ether (EMD Millipore), and ethanol (KOPTEC). All NMR solvents were purchased from Cambridge Isotope Laboratories. NMR spectra were processed using iNMR Reader, version 5.3.4.

2. Cultivation of *Streptomyces cremeus* NRRL 3241, isolation of cremeomycin, and identification of putative degradation product

Cremeomycin was isolated using a procedure adapted from Bergy and co-workers.[1] The entire procedure was conducted taking extreme care to exclude light by using aluminum foil to cover all working flasks and working with the lights off. For isolation of cremeomycin, 100 mL of fermentation media[1] (20 g/L black strap molasses, 10 g/L cottonseed flour, 5 g/L calcium carbonate, 5 mL/L lard oil, tap water, pH adjusted to ~7) was inoculated with *S. cremeus* from a frozen glycerol stock. The flask was wrapped in tinfoil and incubated at 25 °C with shaking at 220 rpm for 9 days. The culture was then filtered through 14 g of Celite to remove cells. The filtered culture was adjusted to pH 3 using 5% sulfuric acid and was washed with hexanes (2 x 50 mL). The organic phase was discarded and the aqueous layer was extracted with dichloromethane (2 x 100 mL). The crude organic extract contained a mixture of two compounds that could be separated by thin-layer chromatography (TLC) (9:1 dichloromethane/methanol, R_f= 0.4 and 0.2). The dichloromethane extract was concentrated *in vacuo* and then purified by flash chromatography on buffered silica gel (buffered silica gel was made by mixing 10.88 g

KH_2PO_4, 0.109 g Na_2HPO_4, and 150 mL H_2O with 200 g of silica gel, and then heating to 130 °C for two hours). Elution with 99:1 dichloromethane/methanol afforded cremeomycin (5.5 mg, 28.3 μmoles, R_f = 0.4) and 2-hydroxy-4-methoxybenzoic acid (2.5 mg, 14.9 μmoles, R_f = 0.2).

Cremeomycin



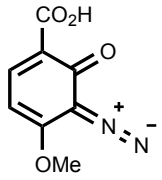
TLC: R_f = 0.4 (silica, 9:1 dichloromethane/methanol)

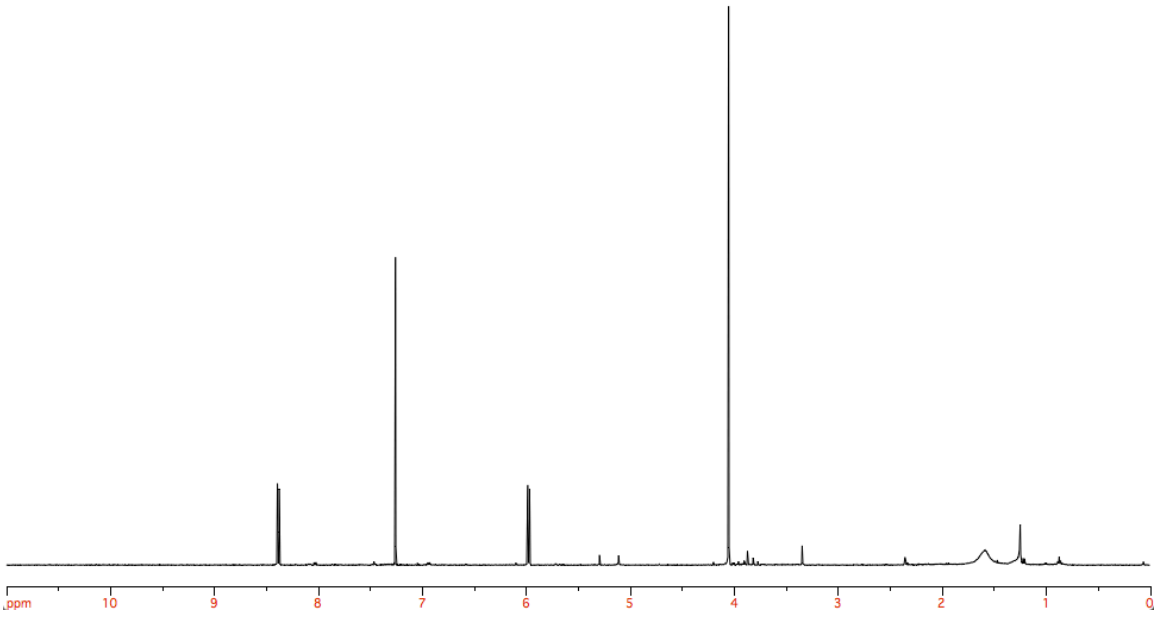
HRMS (ESI): calc'd for $C_8H_7N_2O_4^+$ $[M+H]^+$ = 195.0400; found, 195.0396; calc'd for $C_8H_6N_2O_4Na^+$ $[M+Na]^+$ = 217.0220; found, 217.0215

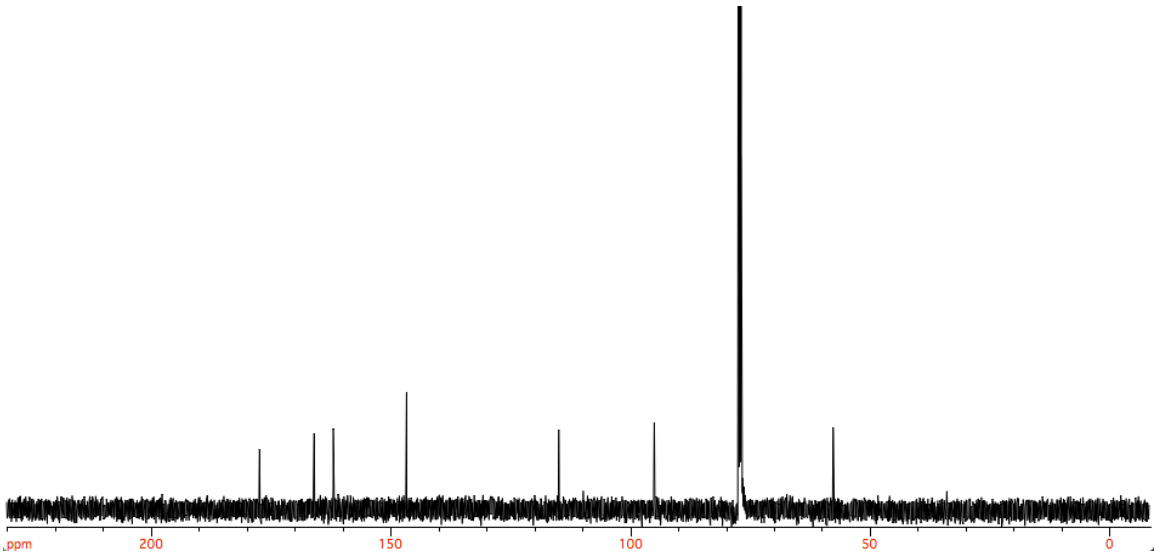
^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.39 (d, *J* = 8.50 Hz, 1H, aromatic C**H**), 5.98 (d, *J* = 8.56 Hz, 1H, aromatic C**H**), 4.06 (s, 3H, OC**H$_3$**)

^{13}C NMR (125 MHz, $CDCl_3$) δ: 177.45 (C2), 166.07 (COOH), 162.07 (C6), 146.77 (C4), 115.01 (C1), 95.09 (C5), 57.78 (CH3)

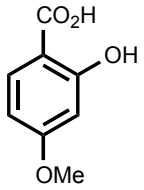
^{1}H and ^{13}C NMR data matched those previously reported.[2] No paramagnetic reagent was added; therefore the diazo carbon was silent in ^{13}C NMR.[2]


CO2H
O
N
N
OMe


ppm
10
9
8
7
6
5
4
3
2
1
0


ppm
200
150
100
50
0

2-hydroxy-4-methoxybenzoic acid (2,4-HMBA)



TLC: R_f = 0.2 (silica, 9:1 dichloromethane/methanol)

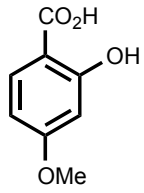
HRMS (ESI): calc'd for $C_8H_7O_4^-$ $[M-H]^-$ = 167.0350; found, 167.0351

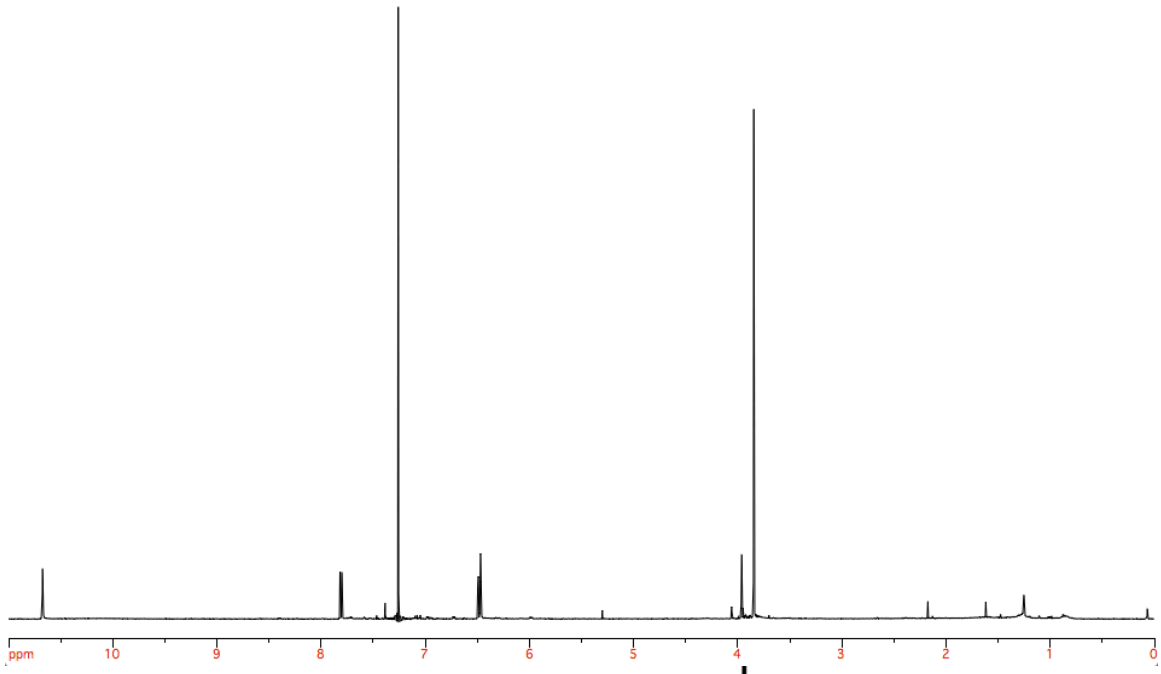
^{1}H NMR (500 MHz, $CDCl_3$) δ: 10.64 (s, 1H, COO**H**), 7.81 (d, *J* = 8.76 Hz, 1H, aromatic C**H**), 6.50 – 6.46 (m, 2H, aromatic C**H**), 3.85 (s, 3H, OC**H$_3$**)

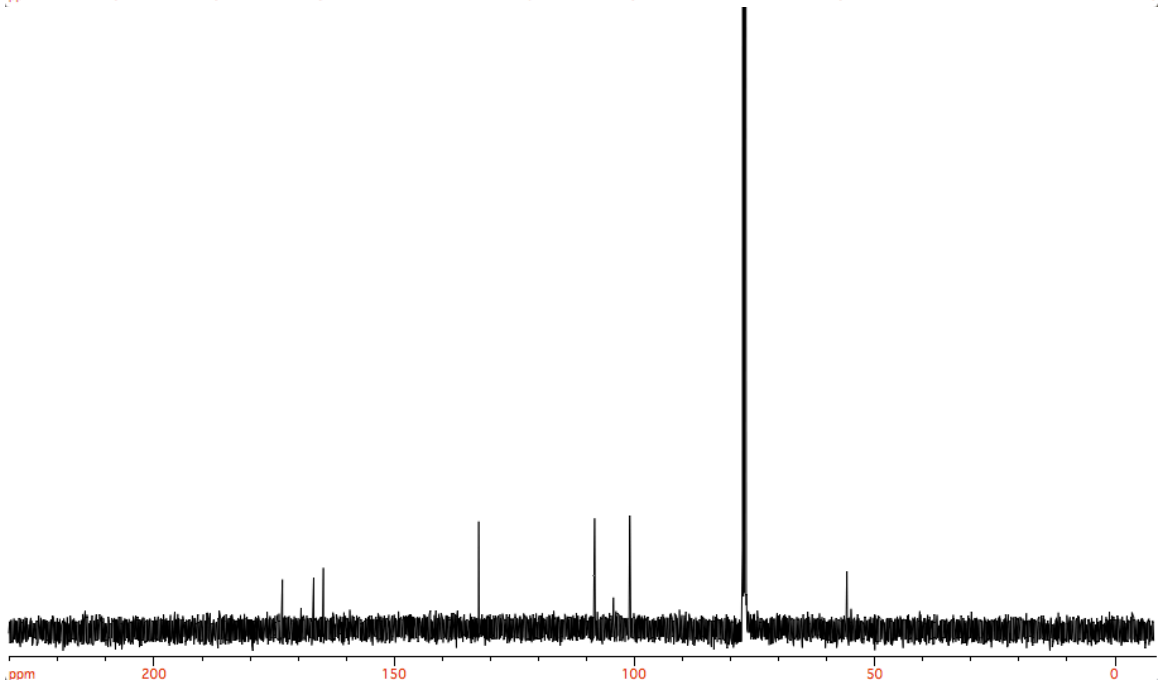
^{13}C NMR (125 MHz, $CDCl_3$) δ: 173.29 (COOH), 166.79 (C4), 164.76 (C2), 132.44 (C6) 108.32 (C1), 104.39 (C5), 101.00 (C3), 55.82 (CH3)

^{1}H and ^{13}C NMR data matched those previously reported.[3]

Further support for this structural assignment was provided in subsequent experiments in which *S. cremeus* crude ethyl acetate extracts were analyzed by LC-MS and LC-MS/MS and compared to a commercial standard of 2-hydroxy-4-methoxybenzoic acid.


CO2H
OH
OMe


ppm
10
9
8
7
6
5
4
3
2
1
0


ppm
200
150
100
50
0

3. Degradation of cremeomycin into 2-hydroxy-4-methoxybenzoic acid

2-Hydroxy-4-methoxybenzoic acid (2,4,-HMBA) was hypothesized to arise from the degradation of cremeomycin via the reduction of the diazo group. A sample of cremeomycin isolated from the protocol above was incubated in sterile fermentation media at 28 °C protected from light. At the indicated time points, 100 uL aliquots were taken and stored at – 80 °C. Samples were then centrifuged at 16,000 x g for 15 min before the supernatant was analyzed by HPLC. A Dionex PolarAdvantage column was used with a flow rate of 0.2 mL/min using 0.1% TFA in water as mobile phase A and 0.1% TFA in acetonitrile as mobile phase B. The following gradient was applied: 0 – 25 min: 0-100% B, 25 – 28 min: 100% B isocratic, 28 – 29 min: 100-0% B, 29 – 37 min: 0% B isocratic. Absorption was monitored using a diode array detector at 254 nm. Cremeomycin is shown to degrade to 2,4-HMBA over time in fermentation media protected from light (Figure S1).

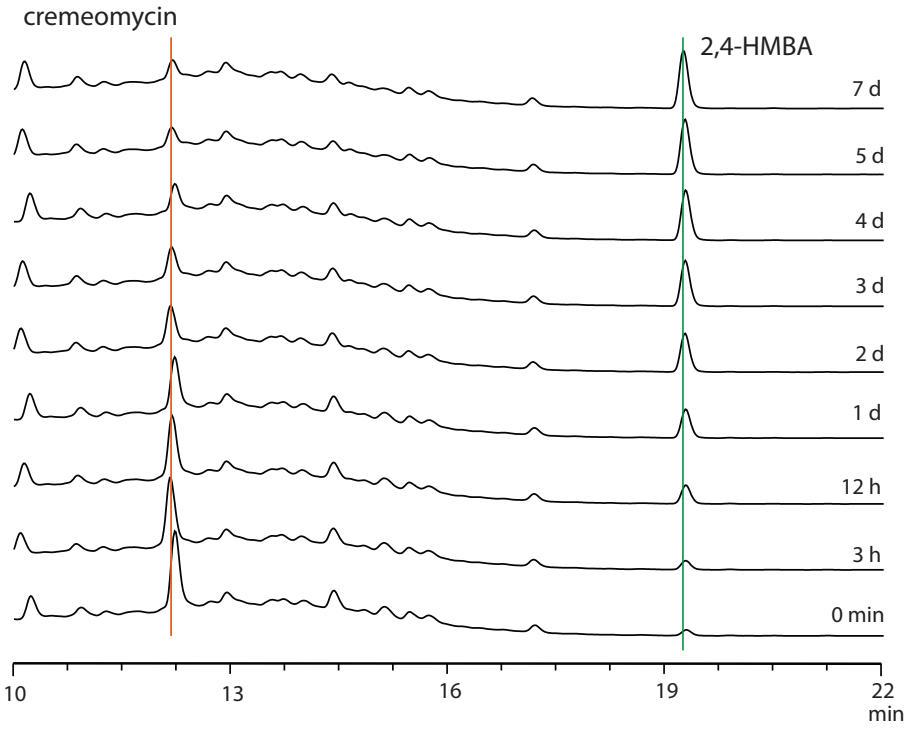


Figure S1. HPLC time course assay of cremeomycin degradation in fermentation media.

4. Identification of a 3,4-AHBA synthase homolog in *S. cremeus* NRRL 3241

S. cremeus NRRL 3241 was cultivated in YM media (Yeast-Malt media contained 10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract) at 28 °C with shaking at 275 rpm for 7 days. Genomic DNA was isolated from the cell pellet using the UltraClean Microbial DNA Isolation Kit (MoBio). Deviations from the manufacturer's protocol were as follows: the lysis step involved heating the MicroBead Tube for 10 minutes at 70 °C followed by vortexing at maximum speed for 10 minutes; 4 μL of RNAase A (100 mg/ml) was added to the MicroBead tube after heating and vortexing and the tubes were then incubated at room temperature for 2 minutes; an additional ethanol wash step was performed prior to elution of the DNA.

Degenerate PCR was used to amplify a fragment of potential 3-amino-4-hydroxybenzoic acid (3,4-AHBA) synthase genes from *S. cremeus* NRRL 3241 genomic DNA using a previously reported primer pair.[4] The PCR reaction contained 25 μL of 2X Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs), 5 μL of genomic DNA template, 1.5 μL DMSO, and 25 pmoles of each primer in a total volume of 50 μL. Thermocycling was carried out in a MyCycler gradient cycler (Bio-Rad) using the following parameters: denaturation for 30 sec at 98 °C, followed by 35 cycles of 10 sec at 98 °C, 30 sec at 71 °C, 20 sec at 72 °C, and a final extension time of 10 min at 72 °C. Analysis of the PCR reaction using agarose gel electrophoresis with ethidium bromide staining revealed a band of the expected size (~ 290 bp). The reaction was purified using agarose gel electrophoresis and the 290 bp gel fragment was excised and extracted from the gel (Illustra GFX kit). The resulting PCR fragment was ligated into the pCR-Blunt II-TOPO vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and the ligation reaction was used to transform *Escherichia coli* TOP10 cells (Invitrogen) according to the manufacturer's protocol. 100 μL of the transformation was plated onto a Luria-Burtani (LB) agar plate containing kanamycin (50 μg/mL) and incubated at 37 °C overnight. Four colonies were used to individually inoculate 5 mL of overnight cultures of LB media supplemented with kanamycin (50 μg/mL). Plasmid DNA was isolated from each overnight culture and the identity of each plasmid insert was confirmed via sequencing. The translated sequence of the 284 bp insert displayed high similarity to the

amino acid sequences of other 3,4-AHBA synthase homologs (GriH, NspH, PtnB2, and PtmB2; 87.2%, 86.2%, 84.0% and 84% percent identity, respectively)

5. *S. cremeus* NRRL 3241 genome sequencing and identification of the putative cremeomycin biosynthetic gene cluster

Library construction from genomic DNA, sequencing, and assembly were performed by Cofactor Genomics (St. Louis, MO). Next-generation sequencing used a combination of Roche 454 long reads and Illumina reads of a short-insert paired-end library and a long-insert mate-pair library. Assembly using Newbler, SOAPdenovo, and Minimus2 resulted in 7.64 MB of non-redundant sequence distributed over 80 scaffolds (N50: 152767 bp).

The assembled data were converted into a local BLAST database using Geneious. A BLAST search using the 284 bp 3,4-AHBA synthase homolog fragment identified through degenerate PCR revealed the location of this sequence on scaffold 28. Open reading frames (ORFs) on scaffold 28 were detected using a combination of BLASTx searches and FGENESB (Softberry) with *Streptomyces coelicolor* A3(2) as the reference organism. Scaffold 28 contained a total of 356 ORFs. The boundaries of the putative cremeomycin biosynthetic gene cluster were proposed based on gene synteny with several other *Streptomyces* strains, particularly *Streptomyces davawensis* JCM 4913, that are not known to produce cremeomycin. Specifically, *S. davawensis* JCM 4913 shared very high levels of synteny with *S. cremeus* NRRL 3241 on either side of an 18 kb region surrounding the 3,4-AHBA synthase homolog. *S. davawensis* JCM 4913 lacked all genes within this 18 kb region. This region was designated the *cre* gene cluster and was deposited into GenBank (accession number = KT381192).

Annotation of all ORFs in the *cre* gene cluster is found in Table S1. Detailed bioinformatic analysis and prediction of the encoded functions of these genes were performed using a combination of homology searching using pBLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch &LINK_LOC=blasthome), conserved domain prediction using the Conserved Domains

Database at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and homology detection and structure prediction using HHPred (http://toolkit.tuebingen.mpg.de/hhpred). Unsequenced regions within the *cre* gene cluster resulting from the initial Illumina and Roche 454 sequencing efforts were sequenced by PCR amplification from genomic DNA, cloning of PCR products, and Sanger sequencing of inserts.

Table S1. ORFs in the *S. cremeus* NRRL 3241 cremeomycin biosynthetic gene cluster and predicted function

Protein	Size, aa	Predicted function	Closest homolog	Accession number	% Identity/ similarity
CreA	365	oxidoreductase	BN6_34700, *Saccharothrix espanaensis* DSM 44229	YP_007037641.1	37/47
CreB	399	cupin protein	Namu_306, *Nakamurella multipartite* DSM 44233	YP_003202389.1	41/55
CreC	467	major facilitator superfamily protein	Cwoe_3061, *Conexibacter woesei* DSM 14684	YP_003394855.1	34/48
CreD	476	3-carboxymuconate cycloisomerase	Saci8_010100025929, *Streptomyces acidiscabies* 84-104	ZP_10453771.1	64/70
CreE	666	oxidoreductase	SSOG_07668, *Streptomyces hygroscopicus* ATCC 53653	ZP_07299585.1	62/71
CreF	196	SARP family regulator	AknI, *Streptomyces galilaeus*	AAF70113.1	53/68
CreG	249	regulatory protein	SRIM_39728, *Streptomyces rimosus subsp. rimosus* ATCC 10970	ZP_20970175.1	51/66
CreH	367	3,4-AHBA synthase	SSOG_07662, *Streptomyces hygroscopicus* ATCC 53653	ZP_07299579.1	87/93
CreI	271	aldolase	SSOG_07661, *Streptomyces hygroscopicus* ATCC 53653	ZP_07299578.1	70/80
CreJ	159	BadM/Rrf2 transcriptional repressor	O31_010600, *Nocardia brasiliensis* ATCC 700358	YP_006807055.1	68/79
CreK	431	Major facilitator superfamily protein	O31_010605, *Nocardia brasiliensis* ATCC 700358	YP_006807056.1	74/82
CreL	394	flavin monooxygenase	PtnB3, *Streptomyces platensis*	ADD82995.1	52/62
CreM	553	long-chain fatty acid CoA ligase	SACE_4925, *Saccharopolyspora erythraea* NRRL 2338	YP_001107116.1	63/74
CreN	365	SAM-dependent methyltransferase	SACE_4031, *Saccharopolyspora erythraea* NRRL 2338	YP_001106227.1	56/68

6. Cloning of *cre* cluster into pCR-Blunt II-TOPO vector to generate pCre plasmid

A genomic library of *Streptomyces cremeus* NRRL 3241 was constructed by using the CopyRight v2.0 BAC Cloning Kit according to manufacturer's protocol. Three pairs of primers were used to screen the genomic library and two BAC clones, 15F11 and 15G3, were found to collectively cover the entire 14 ORF region (*creA* – *creN*) of the putative *cre* biosynthetic gene cluster. To construct the intact *cre* gene cluster into a single plasmid, a 13.6 kb BamHI fragment of 15F11 was first cloned into the same site of pCR-Blunt II-TOPO to generate pW-51. A 8.0 kb BglII and PstI fragment of 15G3 was then ligated into the same BglII and PstI sites of pW-51 to generate pW-52 which harbors the entire *cre* biosynthetic gene cluster (*creA* – *creN*) with no other intact ORFs either upstream or downstream of the 14 ORFs. To allow for chromosomal integration of the *cre* gene cluster within *S. lividans* TK-64, pW-52 was retrofitted with the *int-attP* cassette. A 2.6 kb PstI fragment from pIJ8630 was cloned into pW-52 to generate pCre.

7. Heterologous expression of the *cre* cluster in *Streptomyces lividans* TK-64 and comparison to *S. cremeus* NRRL 3241

The plasmid pCre was introduced into *S. lividans* TK-64 via standard protoplast transformation using kanamycin (200 ug/mL) resistance as a selection marker to obtain the strain *S. lividans* TK-64::*cre*. A typical heterologous expression involved inoculating 50 mL of ISP1 media (containing 200 ug/mL kanamycin) with fresh spores of *S. lividans* TK-64::*cre* from a plate and allowing it to incubate at 28 °C for 3-4 days with shaking at 220 rpm to obtain a starter culture. This starter culture was then used to inoculate 100 mL of ISP1 media (1:50 dilution) containing 200 ug/mL kanamycin. The culture flasks were wrapped in tinfoil and allowed to incubate at 28 °C with shaking at 220 rpm for 7 days. For experiments with *S. cremeus* a 50 mL starter culture in fermentation media was prepared by inoculating fresh spores from a plate and allowing them to incubate at 28 °C for 3-4 days with shaking at 220 rpm. This starter culture was then used to inoculate 100 mL of fermentation media (1:50 dilution). The culture flasks were wrapped in tinfoil and allowed to incubate at 28 °C with shaking at 220 rpm for 7 days. For the comparison of

titers of cremeomycin and degradation product from *S. lividans* TK-64::*cre* and *S. cremeus* NRRL 3241, the strains were cultured in triplicate. (Figure S6, Table S2).

All subsequent steps were performed with attention to reduce sample exposure to light - all containers and glassware covered in tinfoil. After 7 days the cultures were centrifuged (13,000 x *g* for 30 min) and the clarified supernatant was transferred to a separatory funnel. The supernatant was adjusted to pH ~2-3 with 5% sulfuric acid and then extracted with 100 mL of ethyl acetate. The ethyl acetate was concentrated and the residue was redissolved in a methanol/water mixture (1:9) and lyophilized. The lyophilized residue was redissolved in 500 μL of methanol and centrifuged (13,000 x g, 15 min), and the supernatant was submitted for HRMS LC-MS and LC-MS/MS analysis.

Cremeomycin production by *S. lividans* TK-64::*cre* was confirmed by comparison to an authentic standard of cremeomycin isolated from *S. cremeus* by LC-MS (Figure S2) and LC-MS/MS (Figure S3). 2,4-HMBA production by *S. lividans* TK-64::*cre* was confirmed by comparison to an authentic commercial standard of 2,4-HBMA (Sigma) by LC-MS (Figure S4) and LC-MS/MS (Figure S5). Yields from cultures were determined using a standard curve of cremeomycin and 2,4-HMBA standards.

Comparison of cremeomycin and 2,4-HMBA production by *S. cremeus* and *S. lividans* TK-64::*cre* revealed reduced titers in the heterologous host (Figure S6 & Table S2). This observation is perhaps consistent with the known difficulties associated with heterologous production of diazo natural products, and more generally, metabolites containing an N–N bond. [5,6]

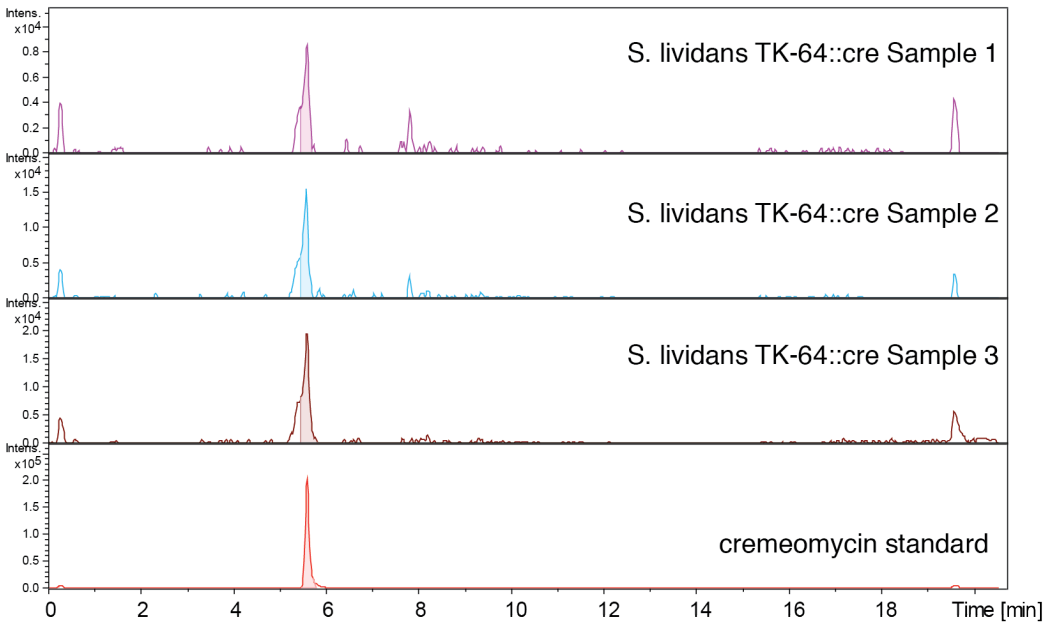


Figure S2. Analysis of *S. lividans* TK-64::*cre* extracts by LC-MS and comparison with cremeomycin standard. EIC of cremeomycin sodium adduct (217.0220) is shown.

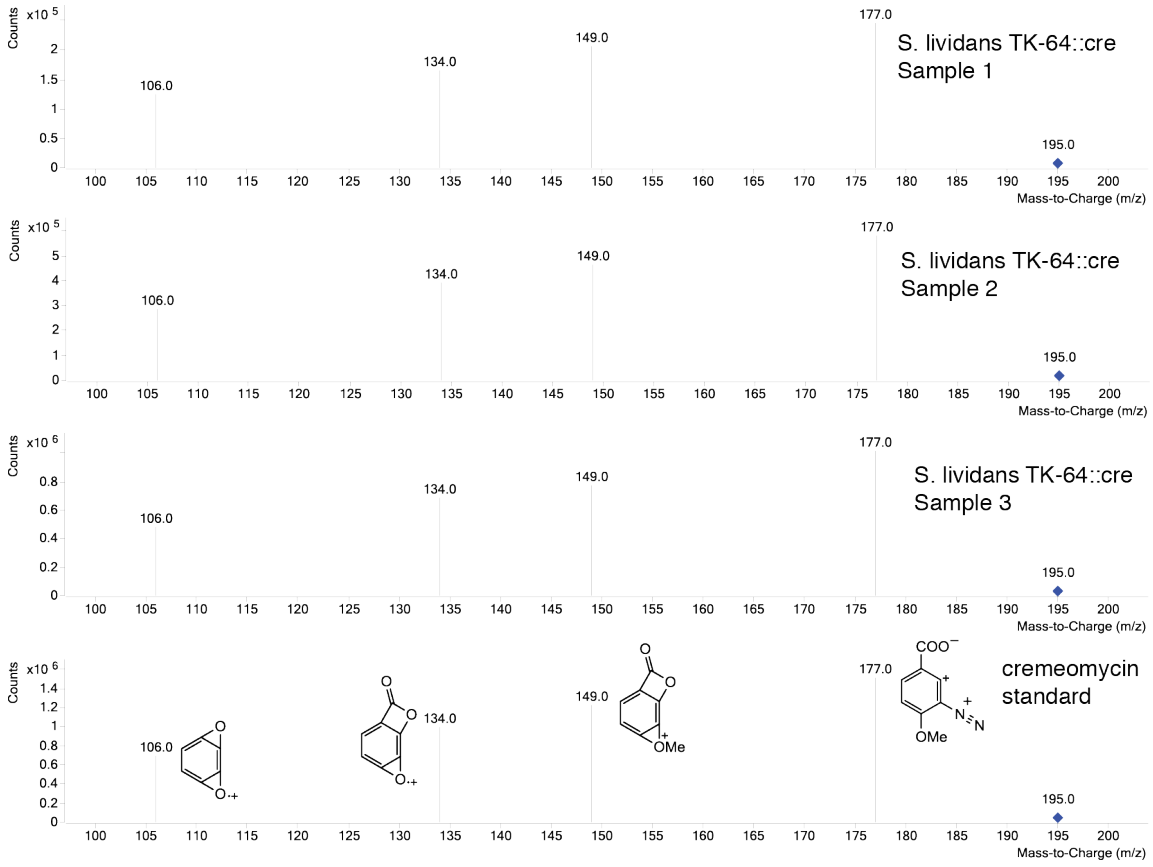


Figure S3. Analysis of *S. lividans* TK-64::*cre* extracts by LC-MS/MS and comparison with cremeomycin standard. Fragment ions are shown.

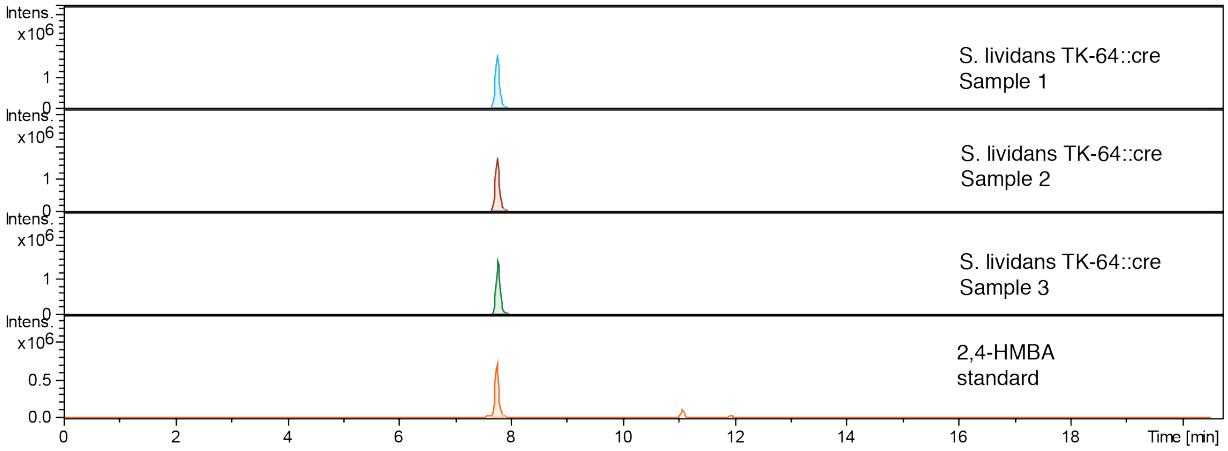


Figure S4. Analysis of *S. lividans* TK-64::*cre* extracts by LC-MS and comparison with 2,4-HMBA commercial (Sigma) authentic standard. EIC of $[M - H^+]^-$ adduct (167.0350) is shown.

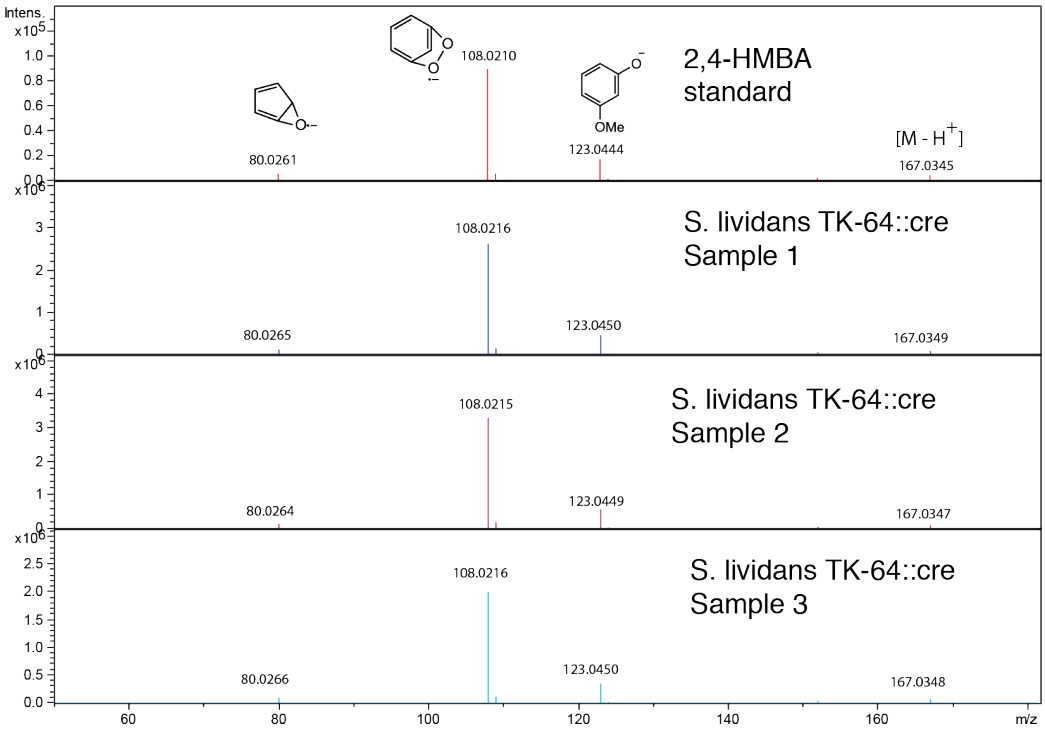


Figure S5. Analysis of *S. lividans* TK-64::*cre* extracts by LC-MS/MS and comparison with 2,4-HMBA commercial (Sigma) authentic standard. Fragment ions are shown.

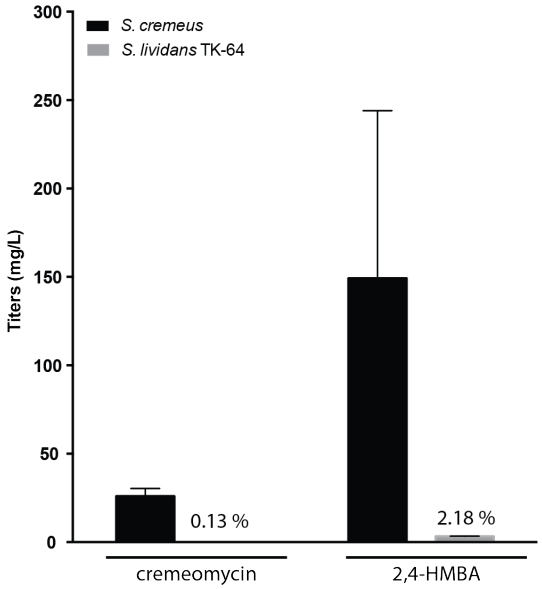


Figure S6. Comparison of titers of cremeomycin and 2,4-HBMA between *S. cremeus* and *S. lividans* TK-64::*cre*. Yield is the average of three independent culturing experiments. Error bars represent the standard deviation.

Table S2. Comparison of titers of cremeomycin and 2,4-HBMA between *S. cremeus* and *S. lividans* TK-64::*cre*. Yield is the average of three independent culturing experiments.

		Yield (mg/L)	St Dev (mg/L)	% yield compared to *S. cremeus*
S. cremeus	cremeomycin	25.9	4.44	-
	2,4-HMBA	149.2	94.8	-
S. lividans* TK-64::*cre	cremeomycin	0.0349	0.0028	0.13
	2,4-HMBA	3.35	0.130	2.18

8. Cloning and heterologous expression of *creHI*

Cloning

Table S3. Oligonucleotides used for cloning *creHI* (restriction sites underlined)

Oligo	Nucleotide sequence
cre-07-for-1	5'-TTATCTCATATGACAGGAACCGCATTACC-3'
cre-06-rev-stop-1	5'-TATACTCGAGCTACTTCTCCAGGCAGAACTC-3'

The genes *creH* and *creI* were cloned into pET-29b. The *creHI* genes were cloned out together from genomic DNA using the primers shown in Table S3. The PCR reaction contained 25 µL of 2X Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs), 1 µL genomic DNA as the template, 25 picomoles of each primer, and 3% DMSO (v/v) in a total volume of 50 µL. Thermocycling was carried out in a MyCycler gradient cycler (Bio-Rad) using the following parameters: denaturation for 30 s at 98 °C, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 71 °C for 30 s, extension at 72 °C for 80 s, and a final extension time for 10 min at 72 °C. The PCR reaction was analyzed by agarose gel electrophoresis with ethidium bromide staining. The band was excised and purified using the Illustra GFX kit. The purified fragment was digested with *NdeI* and *XhoI* (New England Biolabs) for 2.5 h at 37 °C. The digest reaction contained 2 µL MilliQ water, 6 µL NEB Buffer 4 (10X), 6 µL BSA (10X), 3 µL *NdeI* (20 U/µL), 3 µL *XhoI* (20 U/µL), and 40 µL of PCR product. Restriction digests were cleaned using the Illustra GFX kit. The digestion product was ligated into a linearized pET-29b expression vector (*NdeI* and *XhoI*) using T4 DNA ligase (New England Biolabs). The ligation reaction was incubated at room temperature for 2 h and contained 4.5 µL digested insert, 0.5 µL digested vector, 0.7 µL T4 DNA ligase buffer (10X), 0.3 µL MilliQ water, and 1 µL T4 DNA ligase (400 U/µL). 5 µL of ligation mixture was used to transform a single tube of *E. coli* TOP10 cells. The identity of the construct was confirmed by sequencing of purified plasmid DNA.

Heterologous expression

The pET-29b_*creHI* construct was transformed into *E. coli* Tuner cells. An overnight starter culture was made by inoculation of a single colony into LB broth containing 50 µg/mL of kanamycin and incubation at 37 °C on a rotary shaker. This starter culture was used to inoculate (1:100 dilution) 50 mL of M9+glucose supplemented media (34 g/L Na_2HPO_4, 15 g/L KH_2PO_4, 2.5 g/L NaCl, NH_4Cl 5 g/L (M9 salts), 2 mM $MgSO_4$, 100 µM $CaCl_2$, 2.5 µM $MnCl_2$, 1% glycerol (v/v), 1% asparagine (v/v), and 1% glucose (v/v)) with 50 µg/mL of kanamycin. A starter culture of *E. coli* Tuner cells harboring empty pET-29b vector was used to inoculate (1:100) 50 mL of M9+glucose supplemented

media as well. Cultures were incubated with shaking at 175 rpm at 28 °C for 4 h after which the cultures were induced with 50 μM IPTG. The cultures were allowed to incubate for a further 20 h. After 24 h the cultures were centrifuged (13,000 x g for 15 min) and the supernatant was passed through a 0.22 μm filter membrane. A portion of the supernatant was then analyzed by LC-MS.

9. Feeding studies with [^{15}N]-3,4-AHBA and [^{15}N]-3,2,4-AHMBA

S. cremeus NRRL 3241 was grown in 100 mL of fermentation media supplemented with 25 mg (1.6 mM) of [^{15}N]-labeled 3-amino-4-hydroxybenzoic acid ([^{15}N]-3,4-AHBA) or 25 mg (1.3 mM) of 3-amino-2-hydroxy-4-methoxybenzoic acid ([^{15}N]-3,2,4-AHMBA). These cultures were inoculated (1:50) from a starter culture of *S. cremeus* NRRL 3241 in fermentation media that had been started from a freshly sporulated plate. The [^{15}N]-labeled compounds were added at the point of inoculation as solutions in 1 mL of DMSO and filtered through a 0.22 μm filter membrane. Cultures were grown with protection from light (flasks covered in tinfoil) at 28 °C with shaking at 220 rpm.

All subsequent steps were performed with attention to reduce sample exposure to light. After 7 days the cultures were centrifuged (13,000 x g, 30 min) and the supernatant was collected. The supernatant was adjusted to pH ~2-3 with 5% sulfuric acid and extracted with 100 mL of ethyl acetate. The ethyl acetate was concentrated and the residue was redissolved in a methanol/water mixture (1:9) and lyophilized. The lyophilized residue was redissolved in 500 μL of methanol and centrifuged (13,000 x g, 15 min), and the supernatant was submitted for HRMS LC-MS analysis.

Analysis of the extracted ion chromatogram of the cremeomycin sodium adduct and its mass spectra allowed for determination of the extent of labeling. Comparison of the peak intensities of the ~217 Da and ~218 Da masses showed a 40% labeling of cremeomycin for the *S. cremeus* NRRL 3241 culture fed [^{15}N]-3,4-AHBA (Figure S7) and an 86% labeling of cremeomycin for the *S. cremeus* NRRL 3241 culture fed [^{15}N]-3,2,4-AHMBA (Figure S8).

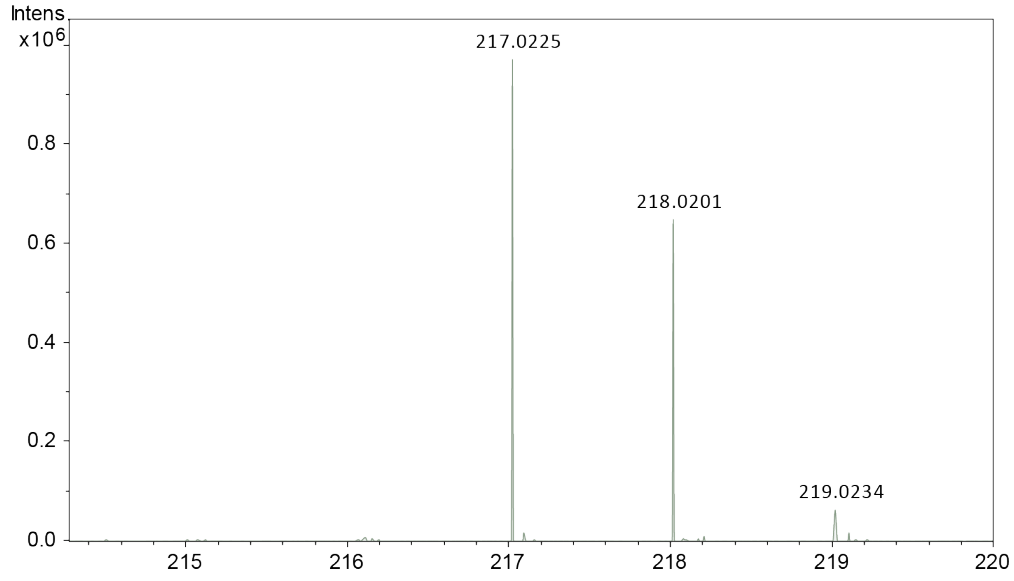


Figure S7. Mass spectrum of cremeomycin sodium adduct from *S. cremeus* NRRL 3241 culture fed with [^{15}N]-3,4-AHBA.

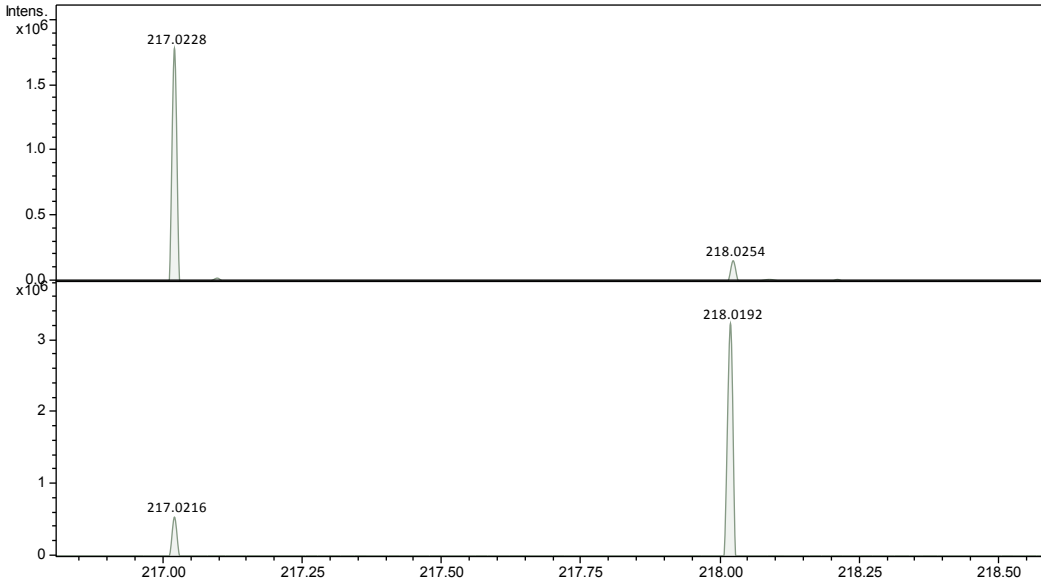


Figure S8. Mass spectrum of cremeomycin sodium adduct from *S. cremeus* NRRL 3241 culture without supplemented (top) and fed with [^{15}N]-3,2,4-AHMBA (bottom).

10. Cloning, overexpression, and purification of CreL and CreN

Cloning

Table S4. Oligonucleotides used for cloning (restriction sites underlined)

Oligo	Nucleotide sequence	ORF amplified
cre-12-for-3	5'-CCCGACCATATGGTTGACCGAGACATCC-3'	*creL* (C-His_6)
cre-12-rev-nostop-3	5'-TATATATACTCGAGGGGCCGGGGGGCGAG-3'	
cre-14-for-3	5'-CTCAACATATGACCGTGCCGGAAAACG-3'	*creN* (C-His_6)
cre-14-rev-nostop-3	5'-TATATACTCGAGCCGGCGCAGGGTGGC-3'	

CreL and *creN* were PCR amplified from *S. cremeus* genomic DNA to afford C-His_6 constructs using the primers shown in Table S4. PCR reactions contained 25 µL 2X Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs), 1 µL genomic DNA, 25 picomoles of each primer, and 3% DMSO (v/v) in a total volume of 50 µL. Thermocycling was carried out in a MyCycler gradient cycler (Bio-Rad) using the following parameters: denaturation for 30 s at 98 °C, followed by 50 cycles of denaturation at 98 °C for 10 sec, annealing at 72 °C for 30 s, extension at 72 °C for 40 s, and a final extension time of 10 min at 72 °C.

The PCR reactions were analyzed by agarose gel electrophoresis with ethidium bromide staining. Bands were excised and purified using Illustra GFX kit. The purified fragments were digested with *NdeI* and *XhoI* (New England Biolabs) for 2.5 h at 37 °C. Digests contained 2 µL MilliQ water, 6 µL NEB Buffer 4 (10X), 6 µL BSA (10X), 3 µL *NdeI* (20 U/µL), 3 µL *XhoI* (20 U/µL), and 40 µL of PCR product. Restriction digests were cleaned using the Illustra GFX kit. Digests were ligated into linearized pET-29b expression vector (*NdeI* and *XhoI*) using T4 DNA ligase (New England Biolabs). Ligations were incubated at room temperature for 2 h and contained 4.5 µL digested insert, 0.5 µL digested vector, 0.7 µL T4 DNA ligase buffer (10X), 0.3 µL MilliQ water, and 1 µL T4 DNA ligase (400 U/µL). 5 µL of the ligation mixtures was used to transform a single tube

of *E. coli* TOP10 cells. The identities of the constructs were confirmed by sequencing of purified plasmid DNA.

Large scale overexpression

The pET-29b/creL-C-His_6 construct was transformed into *E. coli* Rosetta cells (Novagen) and the pET-29b/creN-C-His_6 construct was transformed into *E. coli* Tuner cells (Novagen). A 50 mL starter culture of each strain was inoculated from a frozen cell stock and grown overnight at 37 °C in LB media containing 50 μg/mL of kanamycin and 34 μg/mL of chloramphenicol (chloramphenicol for pET-29b-creL only) with shaking at 175 rpm. The overnight cultures were used to inoculate 2 L of LB media (1:100 dilution) containing 50 μg/mL of kanamycin and 34 μg/mL of chloramphenicol (chloramphenicol for pET-29b-creL only). The cultures were incubated at 37 °C with shaking at 175 rpm for ~2.5 h and then transferred to an incubator at 15 °C with shaking at 175 rpm. At an OD_{600} = 0.5 – 0.6 the cultures were induced with 50 μM IPTG and allowed to continue incubating at 15 °C with shaking at 175 rpm overnight.

Purification

Cells from the 2 L culture were harvested by centrifugation (6,000 rpm x 15 min) and resuspended in 40 mL of lysis buffer (20 mM Tris-HCl pH = 8.5, 500 mM NaCl, 10 mM $MgCl_2$). The cells were lysed by passage through a cell disruptor (Avestin EmulsiFlex-C3) twice at 5,000 – 6,000 psi and the lysate was clarified by centrifugation (15,000 rpm x 30 min). The supernatant was incubated with 2 mL of Ni-NTA resin and 5 mM imidazole for 2 h at 4 °C on a nutating mixer. The mixture was centrifuged (4,000 rpm x 6 min) and the unbound supernatant fraction was discarded. The Ni-NTA resin was transferred to a glass column. Protein was eluted from the column using a stepwise imidazole gradient in elution buffer (20 mM Tris-HCl pH = 8.5, 500 mM NaCl, 10 mM $MgCl_2$, and 5, 25, 50, 75, 100, 125, 150, 200 mM imidazole) while collecting 2 mL fractions – later fractions were yellow in color. SDS-PAGE (4-15% Tris-HCl gel) was used to ascertain the presence and purity of protein in each fraction. Fractions containing the desired protein were combined and dialyzed against 2 L of storage buffer: for creL, 25 mM Tris-SO_4 pH 8.5, 10% glycerol; and for creN, 25 mM Tris-HCl pH 8.5, 50 mM

NaCl, 10% glycerol. Solutions containing protein were frozen in liquid nitrogen and stored at – 80 °C. This procedure afforded a yield of 0.77 mg/L of culture for C-His_6 tagged CreL and final stock concentration of 36 μM. For C-His_6 tagged CreN the yield was 10.3 mg/L of culture and a final stock concentration of 520 μM. (Figure S9).

Cofactor Analysis of CreL

CreL-C-His_6 samples were yellow and purified with bound FAD (Figure S10). For cofactor determination, a sample of CreL-C-His_6 was heated at 95 °C for 5 min to release the cofactor. The sample was centrifuged at 16,000 x g for 15 min to remove precipitated protein and the supernatant was analyzed by LC-MS/MS on an Agilent QQQ and compared to commercial standards of FAD and FMN.

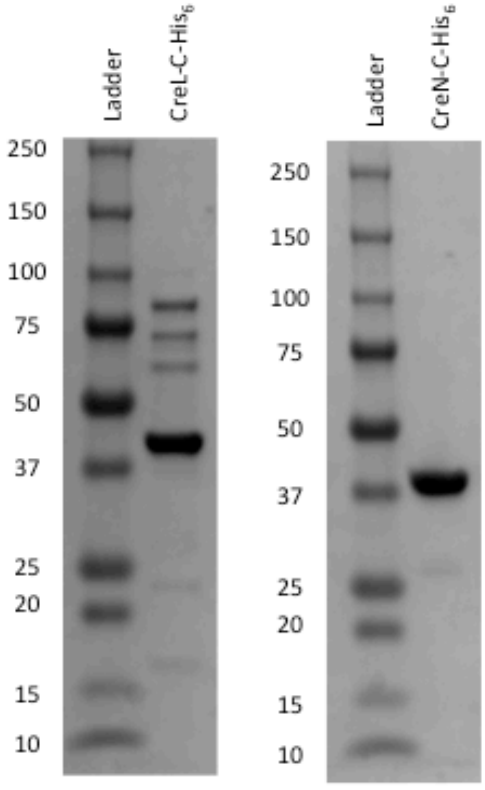


Figure S9. SDS-PAGE of purified C-His_6-tagged CreL (left) and CreN (right). CreL-C-His_6 = 43,882.6 Da and CreN-C-His_6 =40,856.2 Da. 4-15% Tris-HCl gel (BioRad). Ladder = Precision Plus Protein All Blue Molecular Weight Standards (BioRad).

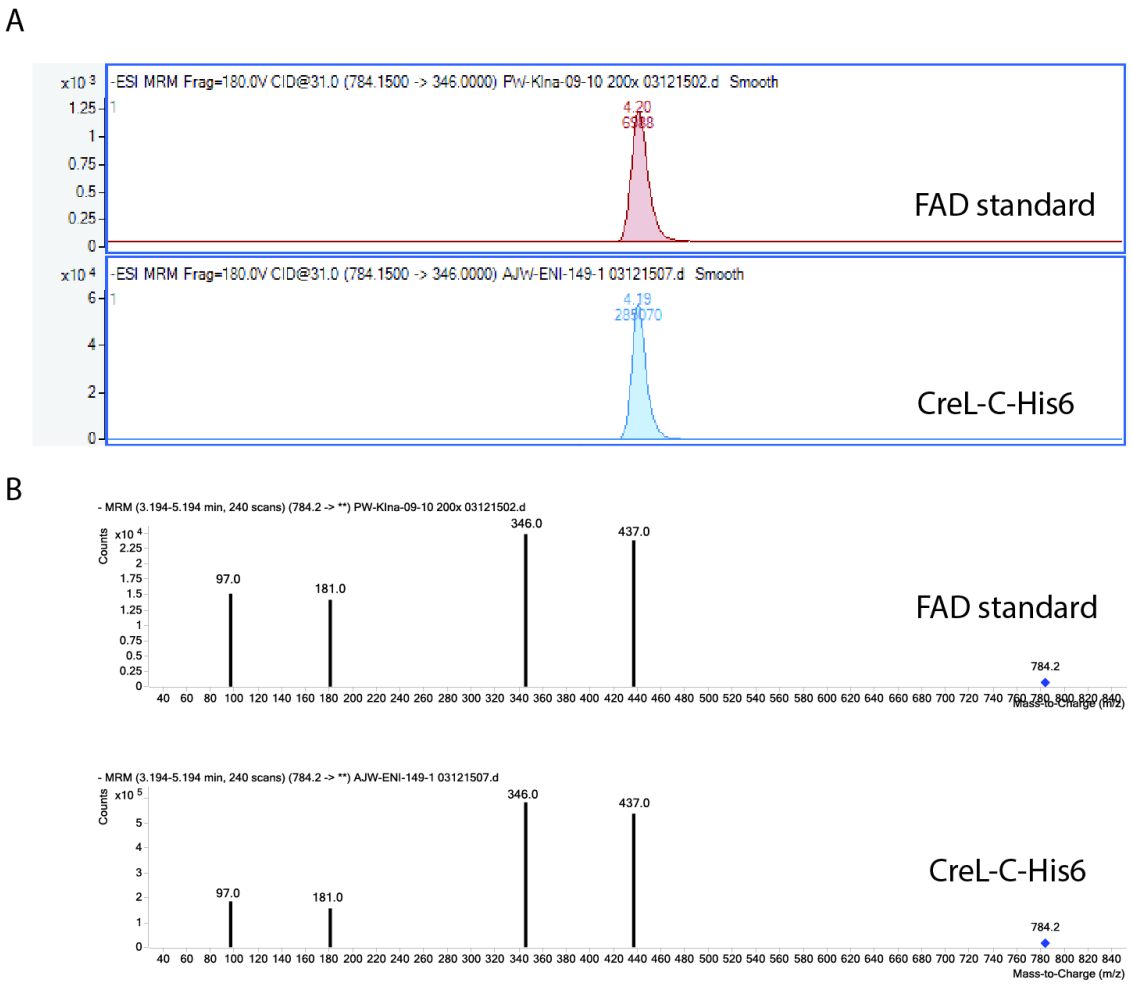


Figure S10. Analysis of CreL-C-His$_6$ heated supernatant by LC-MS/MS reveals presence of flavin adenine dinucleotide (FAD). A) Extracted ion chromatogram of parent ion. B) Fragmentation pattern of daughter ions.

11. Biochemical characterization of CreL and CreN

HPLC assay of CreL hydroxylase activity

For a typical assay, a 300 μL solution containing 75 mM Tris-SO_4 pH 8.0, 2 mM substrate (3-amino-4-hydroxybenzoic acid or 3-amino-4-methoxybenzoic acid), 4 mM NADPH, 5 μM FAD and 2.5 μM CreL-C-His_6 was prepared, mixed, and incubated at room temperature. An aqueous solution containing Tris-SO_4 buffer, substrate, NADPH and FAD was prepared first and vortexed. The enzyme was then added and the solution was gently mixed and incubated at room temperature. Periodically, 50 μL aliquots were removed from the reaction, added to 100 μL of ice-cold methanol, vortexed, and stored at – 20 °C before analysis. The aliquots were then centrifuged (16,000 x g for 15 min) before a portion of the supernatant was analyzed by HPLC. Synthetic and commercial standards were used for verifying the identities of peaks in the traces. A Hypercarb column was used with a flow rate of 0.2 mL/min using 0.1% TFA in water as mobile phase A and 0.1% TFA in acetonitrile as mobile phase B. The following gradient was applied: 0 – 20 min: 0-100% B, 20 – 23 min: 100% B isocratic, 23 – 24 min: 0-100% B, 24 – 30 min: 0% B isocratic. Absorption was monitored using a diode array detector at 254 nm.

HPLC assay of CreN O-methyltransferase activity

For a typical assay, a 450 μL solution containing 75 mM sodium phosphate pH 8.0, 25 mM NaCl, 2 mM substrate (3-amino-2,4-dihydroxybenzoic acid or 3-amino-2-hydroxy-4-methoxybenzoic acid), 4 mM S-adenosylmethionine (SAM), and 10 μM CreN-C-His_6 was prepared, mixed, and incubated at room temperature. An aqueous solution containing sodium phosphate buffer, substrate, and SAM (freshly prepared) was prepared first and vortexed. The enzyme was then added and the solution was gently mixed and incubated at room temperature. Periodically, 50 μL aliquots were removed from the reaction, added to 100 μL of ice-cold methanol, vortexed, and stored at – 20 °C before analysis. The aliquots were centrifuged (16,000 x g for 15 min) before a portion of the supernatant was analyzed by HPLC (Figure S11). Synthetic and commercial standards were used for verifying the identities of peaks in the traces. A Hypercarb column was used with a flow rate of 0.2 mL/min using 0.1% TFA in water as mobile phase A and 0.1% TFA in

acetonitrile as mobile phase B. The following gradient was applied: 0 – 10 min: 0-40% B, 10 – 25 min: 40-60% B, 25 – 26 min: 60-100% B, 26 – 30 min: 100% B isocratic, 30 – 31 min: 100-0% B, 31 – 38 min: 0% B isocratic. Absorption was monitored using a diode array detector at 254 nm.

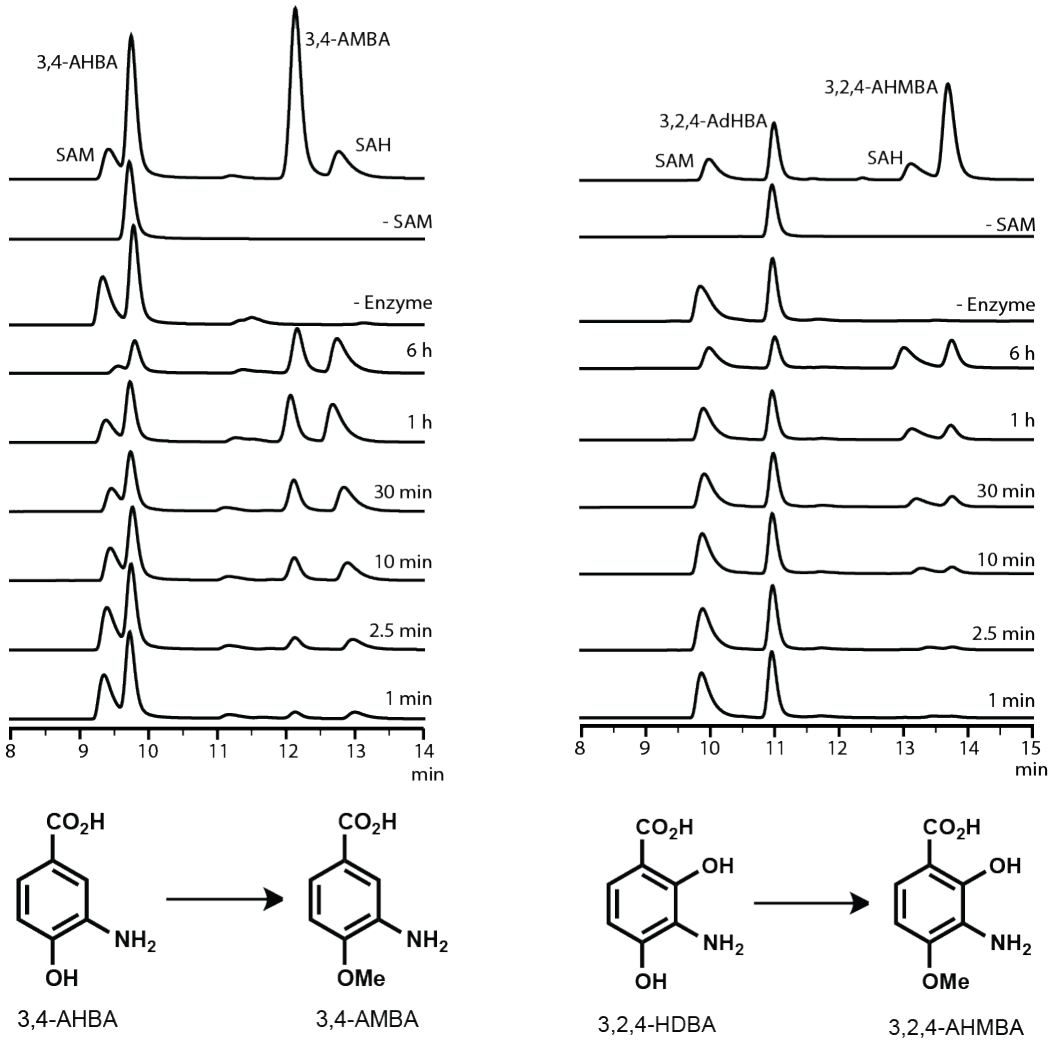
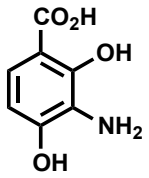


Figure S11. CreN-catalyzed O-methylation *in vitro* assay with (A) 3,4-AHBA and (B) 3,2,4-ADBA shows no selectivity between the two substrates. 3,4-AHBA = 3-amino-4-hydroxybenzoic acid; 3,4-AMBA = 3-amino-4-methoxybenzoic acid; 3,2,4-AHBA = 3-amino-2,4-dihydroxybenzoic acid; 3,2,4-AHMBA = 3-amino-2-hydroxy-4-methoxybenzoic acid; SAM = S-adenosylmethionine; and SAH = S-adenosylhomocysteine.

12. Chemical synthesis procedures and characterization data

3-Amino-2,4-dihydroxybenzoic acid

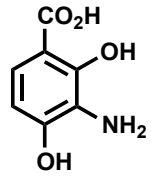


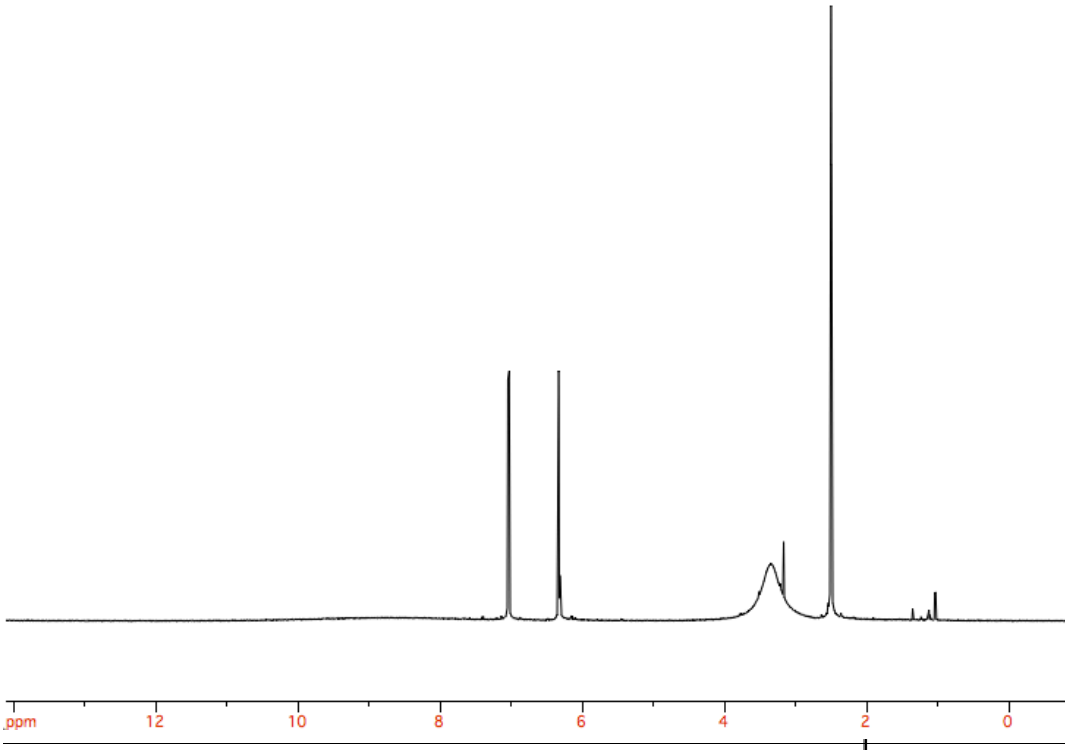
The synthesis of 3-amino-2,4-dihydroxybenzoic acid was accomplished through an adaptation of the previous report by Hayashida *et al*.[7] ^{1}H and ^{13}C NMR data matched those previously reported.

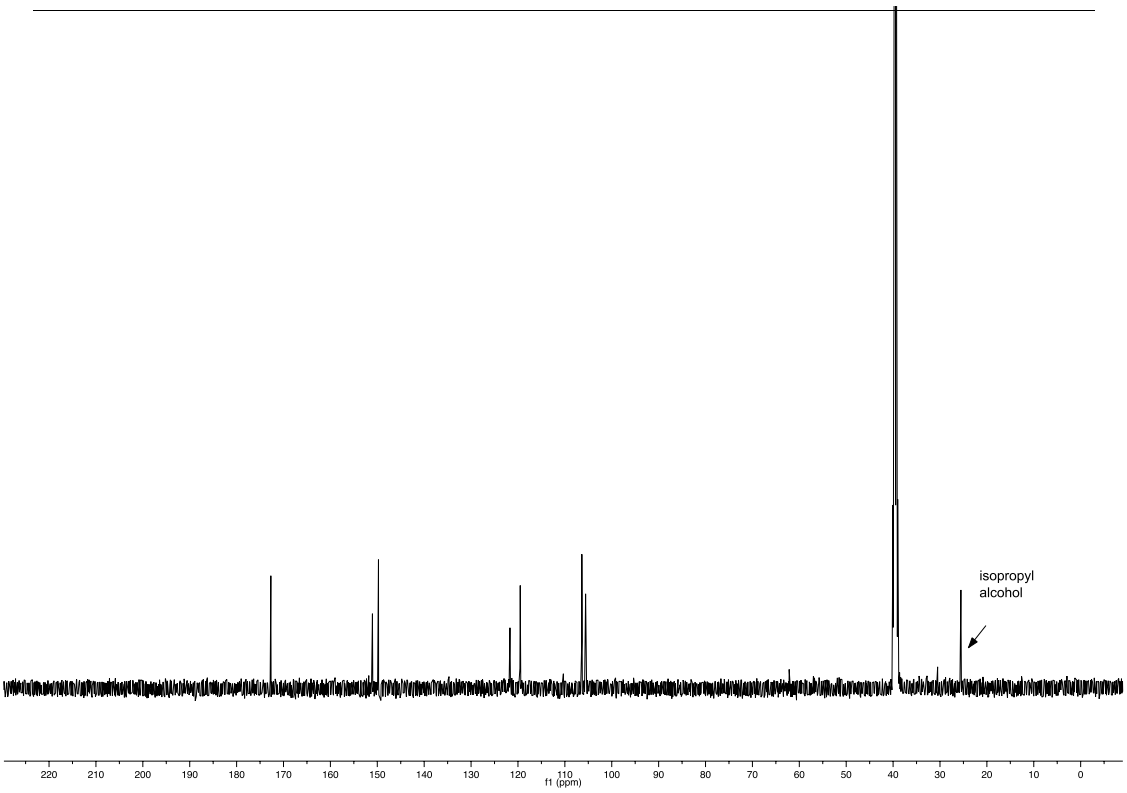
HRMS (ESI): calc'd for $C_7H_8NO_4$, $[M+H]^+$, 170.0448; found, 170.0463

^{1}H NMR (500 MHz, d_6-DMSO) δ: 7.04 (d, J = 8.50 Hz, 1H, aromatic 6-**H**), 6.32 (d, J = 8.65 Hz, 1H, aromatic 5-**H**)

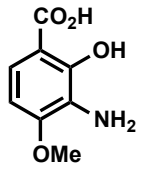
^{13}C NMR (125 MHz, d_6-DMSO) δ: 172.70 (COOH), 151.04 (C4), 149.75 (C2), 121.71 (C6), 119.52 (C3), 106.37 (C5), 105.57 (C1)


CO2H
OH
NH2
OH


ppm
12
10
8
6
4
2
0


isopropyl
alcohol
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

3-Amino-2-hydroxy-4-methoxybenzoic acid

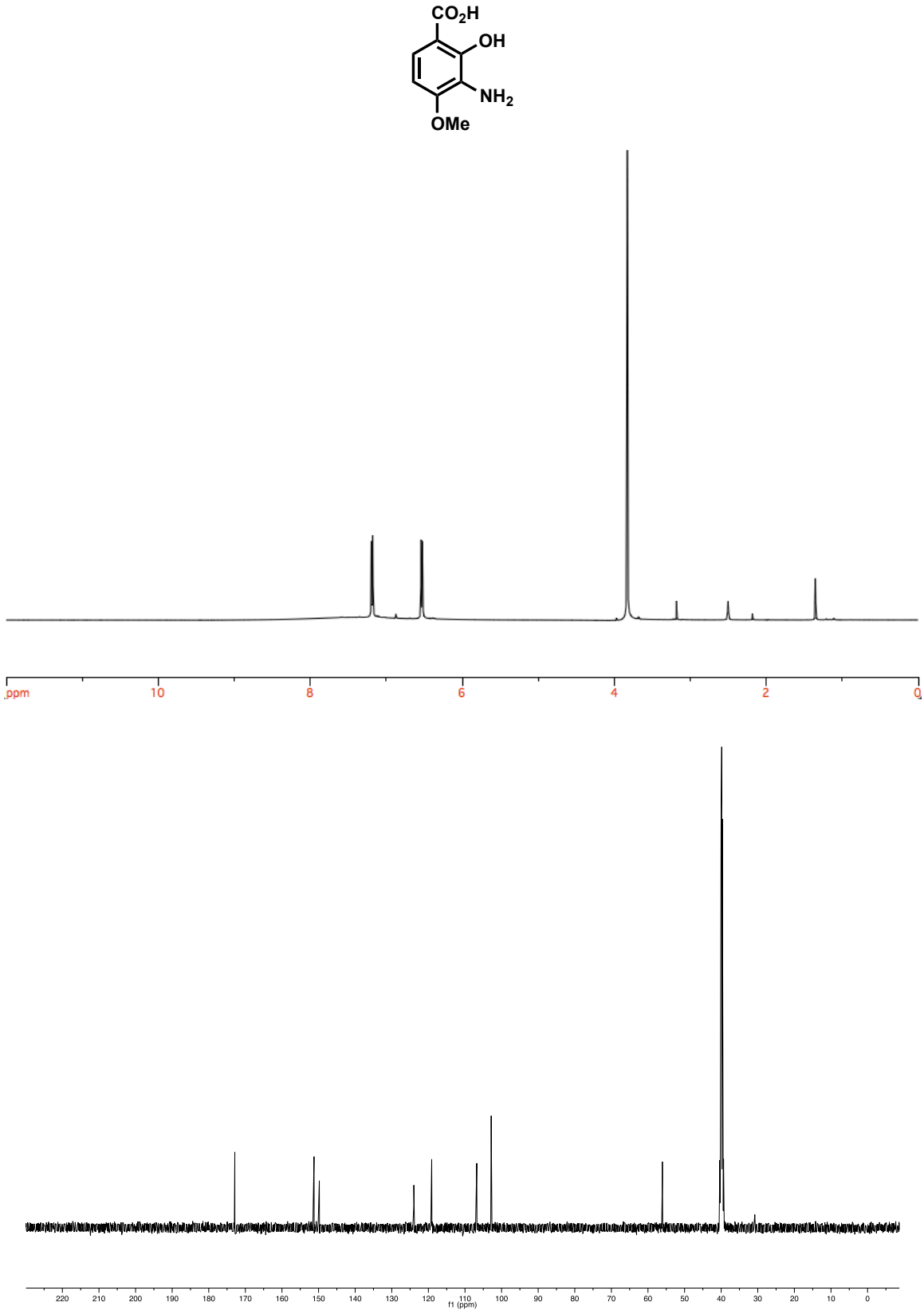


The synthesis of 3-amino-2-hydroxy-4-methoxybenzoic acid was accomplished by following the previous report by Varley *et al*.[8] ^{1}H and ^{13}C NMR data matched those previously reported.

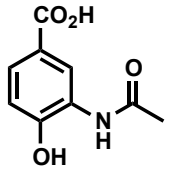
HRMS (ESI): calc'd for $C_8H_{10}NO_4^+$ $[M+H]^+$, 184.0604; found, 184.0609

^{1}H NMR (500 MHz, d_6-DMSO) δ: 7.18 (d, J = 8.80 Hz, 1H, aromatic 6-**H**), 6.53 (d, J = 8.86 Hz, 1H, aromatic 5-**H**), 3.82 (s, 3H, OC**H$_3$**)

^{13}C NMR (125 MHz, d_6-DMSO) δ: 172.55 (COOH), 150.90 (C4), 149.46 (C2), 123.63 (C6), 118.76 (C2), 106.45 (C1), 102.50 (C5), 55.73 (CH3)


CO2H
OH
NH2
OMe
ppm
10
8
6
4
2
0
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

3-Acetamido-4-hydroxybenzoic acid

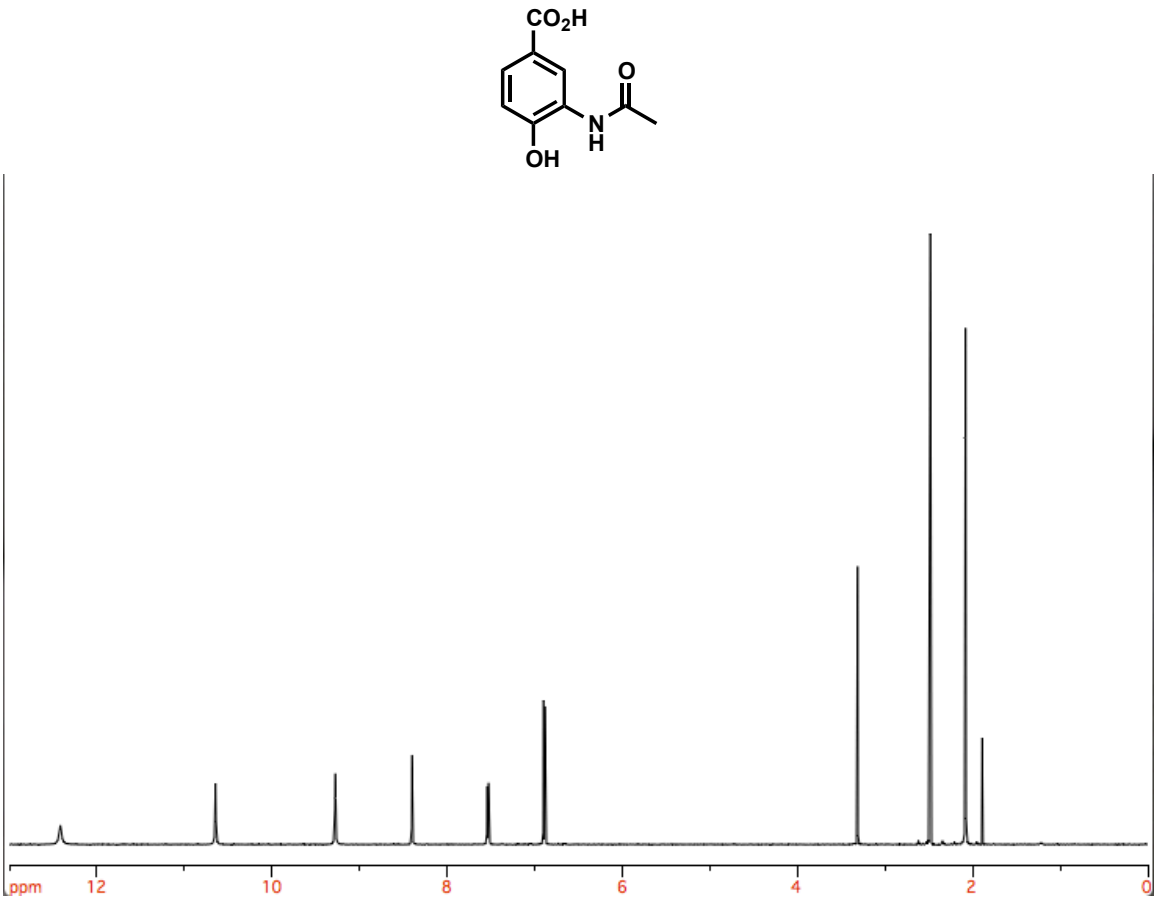


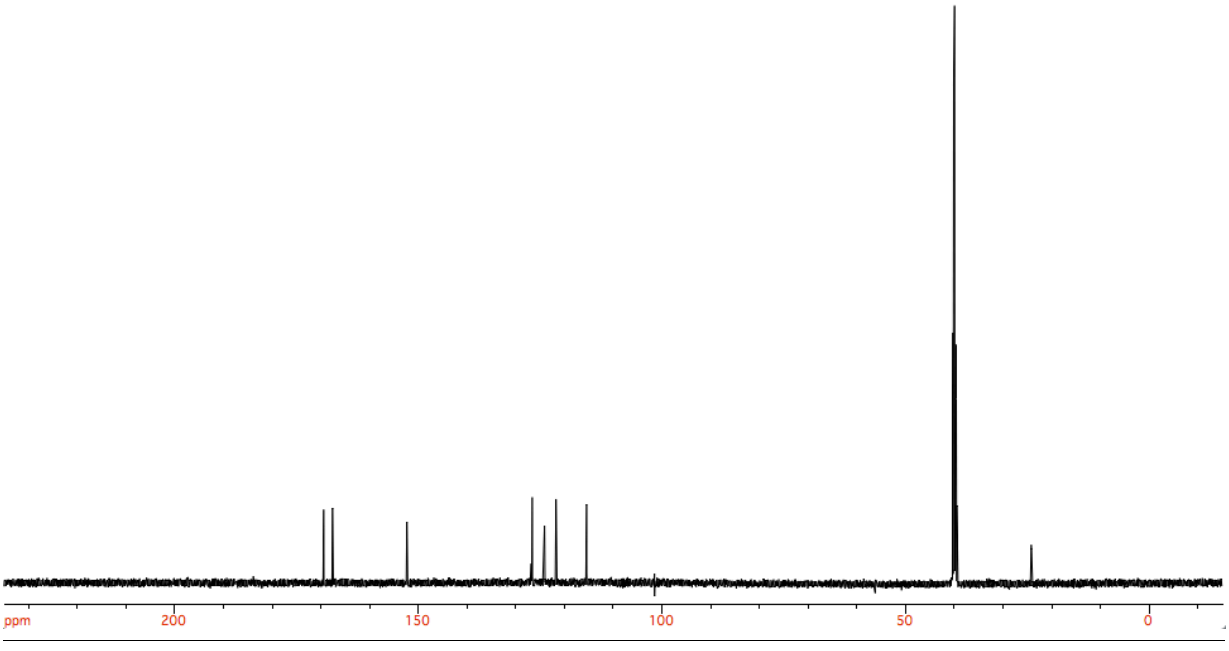
The synthesis of 3-acetamido-4-hydroxybenzoic acid was accomplished by following the previous report by the Mao *et al*.[9] ^{1}H and ^{13}C NMR data matched those previously reported.[10]

HRMS (ESI): calc'd for $C_9H_8NO_4^-$ $[M-H]^-$, 194.0448; found, 194.0456

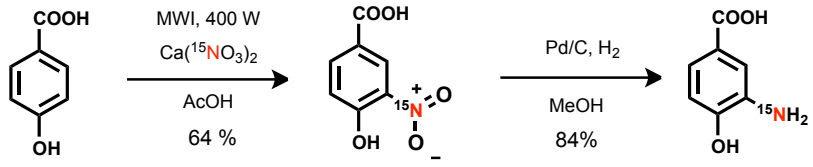
^{1}H NMR (500 MHz, d_6-DMSO) δ: 12.43 (s, 1H, COO**H**), 10.66 (s, 1H, O**H**), 9.29 (s, 1H, N**H**), 8.42 (d, J = 2.07 Hz, 1H, aromatic 2-**H**), 7.55 (dd, J = 8.40, 2.13 Hz, 1H, aromatic 6-**H**). 6.91 (d, J = 8.41 Hz, 1H, aromatic 5-**H**), 2.10 (s, 3H, COC**H$_3$**)

^{13}C NMR (125 MHz, d_6-DMSO) δ: 168.99 (CONH), 167.18 (COOH), 151.91 (C4), 126.38 (C3), 126.19 (C6), 123.68 (C2), 121.31 (C1), 115.03 (C5), 23.74 (CH3)

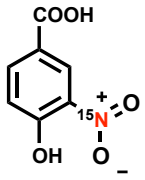

CO2H
O
N
H
OH
ppm
12
10
8
6
4
2
0


ppm
200
150
100
50
0

General scheme for synthesis of [^{15}N]-3-amino-4-hydroxybenzoic acid



[^{15}N]-4-hydroxy-3-nitrobenzoic acid



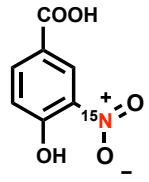
The synthesis of [^{15}N]-4-hydroxy-3-nitrobenzoic acid was accomplished by following the procedure previously reported by Bose *et al* except for the use of [^{15}N]-$Ca(NO_3)_2$.[11]

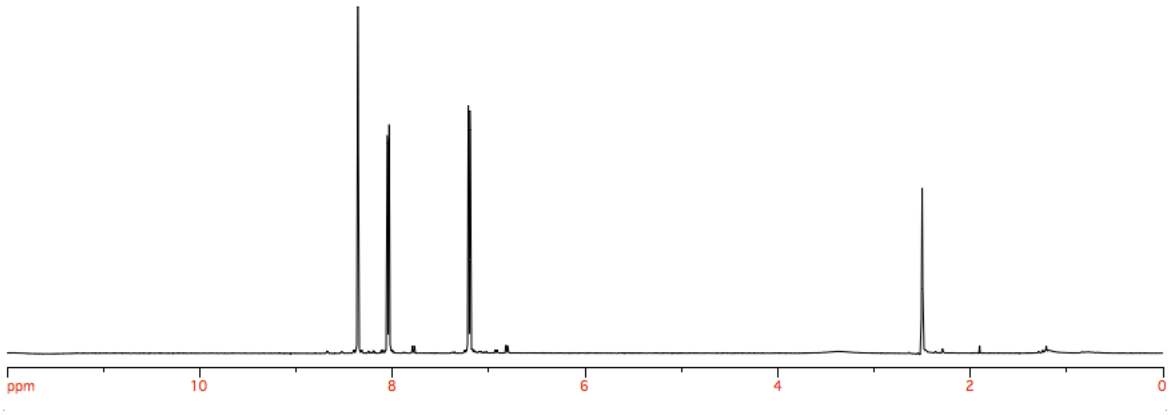
A mixture of 4-hydroxybenzoic acid (600 mg, 4.35 mmol) and [^{15}N]-$Ca(NO_3)_2$ (1445 mg, 8.700 mmol) in 3 mL of glacial acetic acid was heated at 400 MW power in a domestic microwave for 1 min. Three mL of ice-cold water were then added and the reaction mixture was incubated in an ice-water bath for 30 min. The solid was then filtered, washed with 15 mL of ice-cold water, and dried *in vacuo* to afford [^{15}N]-4-hydroxy-3-nitrobenzoic acid (508.9 mg, 2.760 mmol, 64%) as a pale-yellow solid.

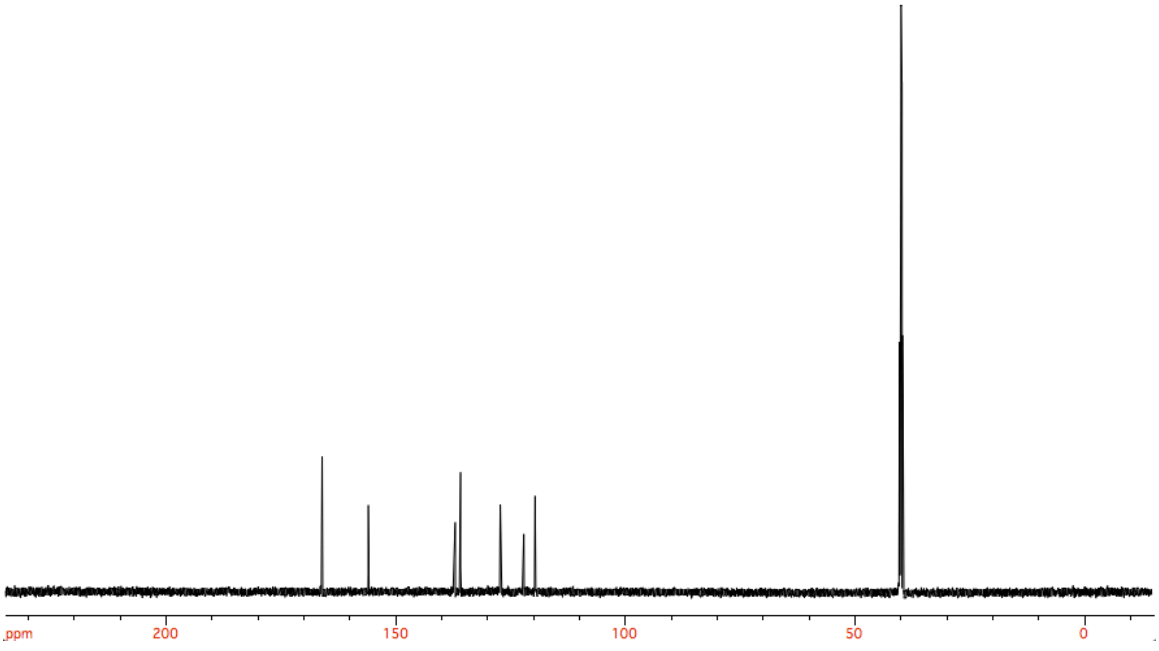
HRMS (ESI): calc'd for $C_7H_4{}^{15}NO_5^-$ $[M-H]^-$, 183.0065; found 183.0063

^{1}H NMR (500 MHz, d_6-DMSO) δ: 8.36 (t, J = 2.30 Hz, 1H, aromatic 2-**H**), 8.04 (dd, J = 8.72, 2.17 Hz, 1H, aromatic 6-**H**), 7.20 (dd, J = 8.71, 0.99 Hz, 1 H, aromatic 5-**H**)

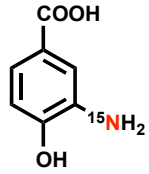
^{13}C NMR (125 MHz, d_6-DMSO) δ: 166.04 (COOH), 155.98 (C4), 137.11 (J = 16.25 Hz, C3), 135.89 (C6), 127.24 (C2), 122.11 (C1), 119.66 (C5)


COOH
OH
15
N
O
O


ppm
10
8
6
4
2
0


ppm
200
150
100
50
0

[^{15}N]-3-amino-4-hydroxybenzoic acid



The synthesis of [^{15}N]-3-amino-4-hydroxybenzoic acid was accomplished by following the procedure previously reported by Xiao *et al* except for the use of ^{15}N-labeled substrate.[12]

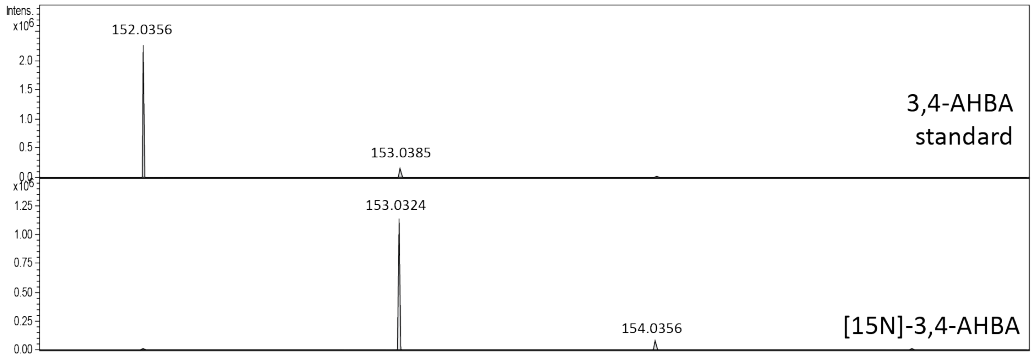
A mixture of [^{15}N]-4-hydroxy-3-nitrobenzoic acid (100 mg, 0.543 mmol) and Pd/C (10 mg, 10 weight %) was stirred in methanol at room temperature under an atmosphere of H_2 overnight. The reaction was filtered through Celite and the filtrate was concentrated *in vacuo* to afford [^{15}N]-3-amino-4-hydroxybenzoic acid (70 mg, 0.454 mmol, 84%) as a dark brown-black solid.

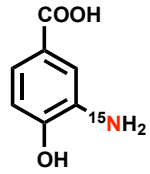
HRMS (ESI): calc'd for $C_7H_6{}^{15}NO_3^-$ $[M-H]^-$, 153.0324; found, 153.0324

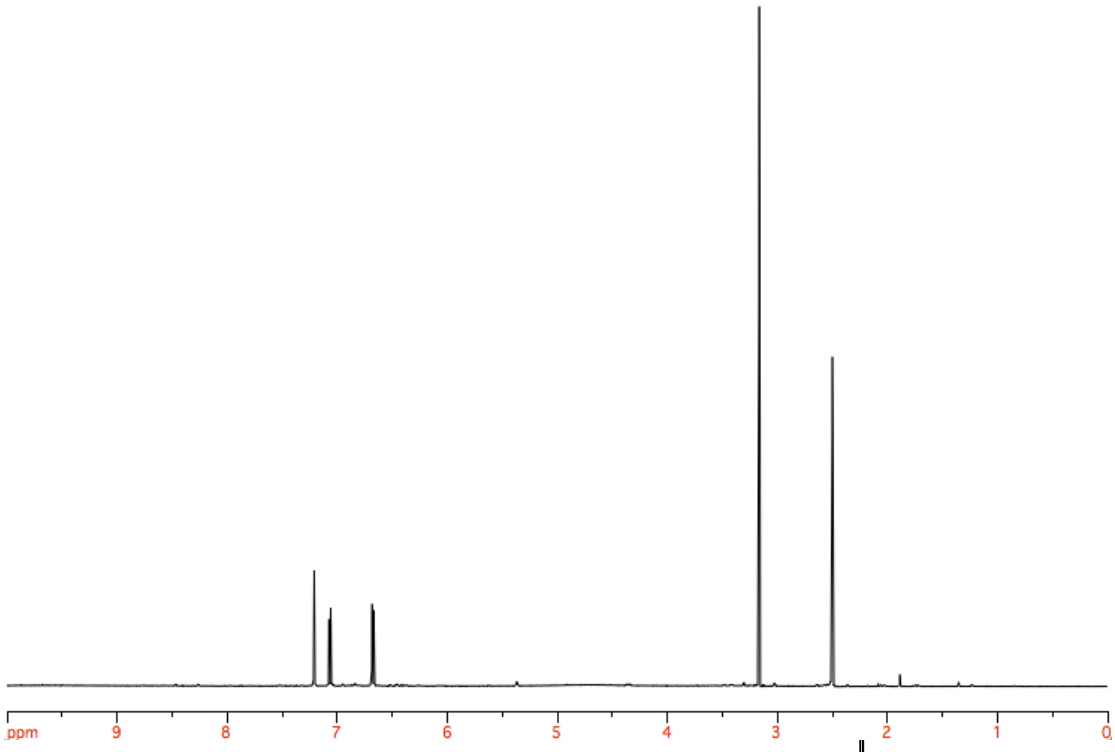
^{1}H NMR (500 MHz, d_6-DMSO) δ: 7.21 (t, J = 2.05 Hz, 1H, aromatic 2-**H**), 7.06 (dd, J = 8.2, 2.1 Hz, 1H, aromatic 6-**H**), 6.67 (d, J = 8.15 Hz, 1H, aromatic 5-**H**)

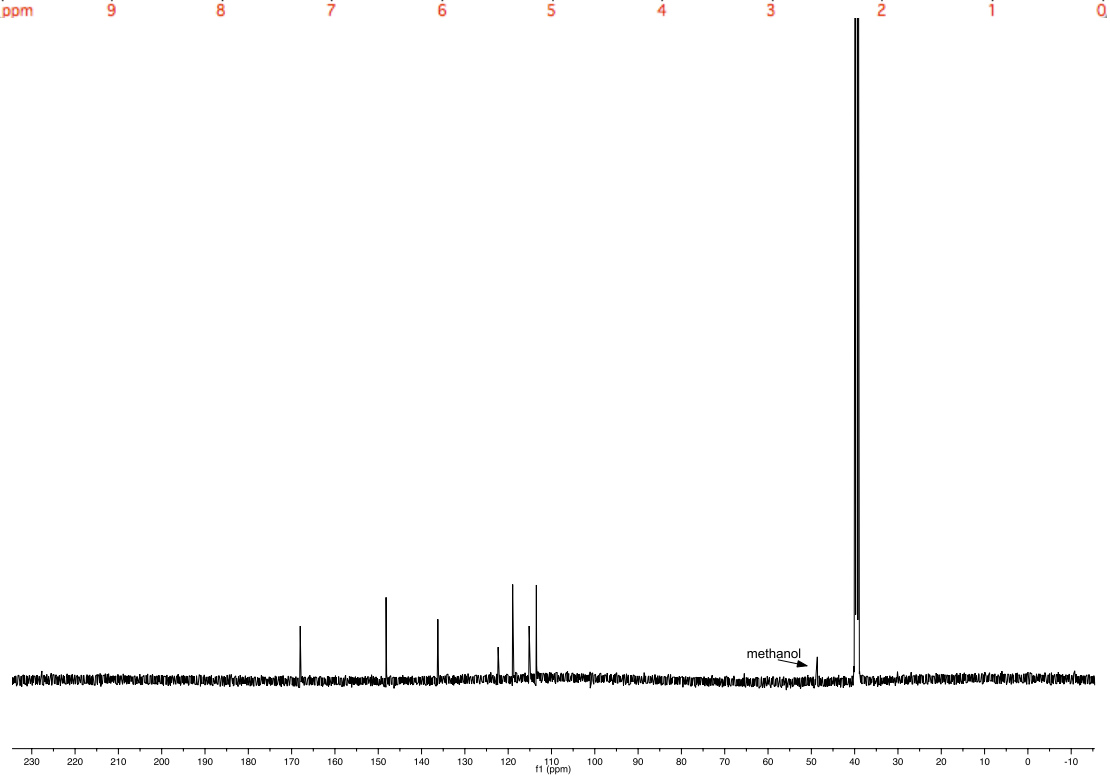
^{13}C NMR (125 MHz, d_6-DMSO) δ: 168.01 (COOH), 148.19 (C4), 136.29 (d, J = 12 Hz, C3), 122.32 (C6), 118.95 (C1), 115.16 (C5), 113.49 (C2)

The sample was 99% enriched in ^{15}N as analyzed by HRMS. MS spectra shown below.

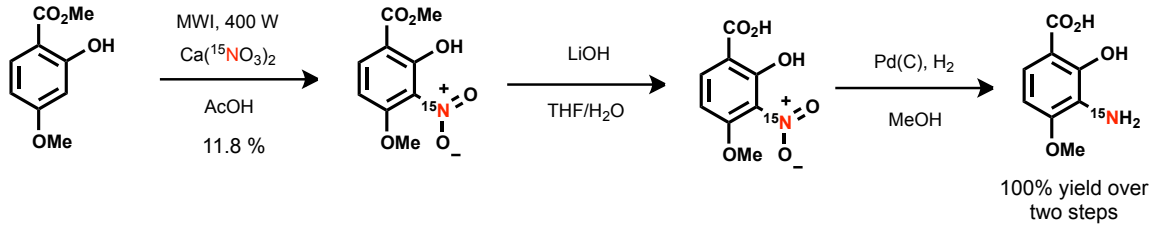



COOH
15NH2
OH

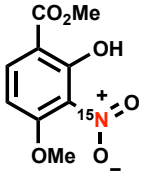

ppm
9
8
7
6
5
4
3
2
1
0


methanol
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

General scheme for synthesis of [^{15}N]-3-amino-2-hydroxy-4-methoxybenzoic acid



Methyl 2-hydroxy-4-methoxy-3-[^{15}N]nitrobenzoate

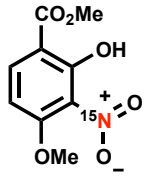


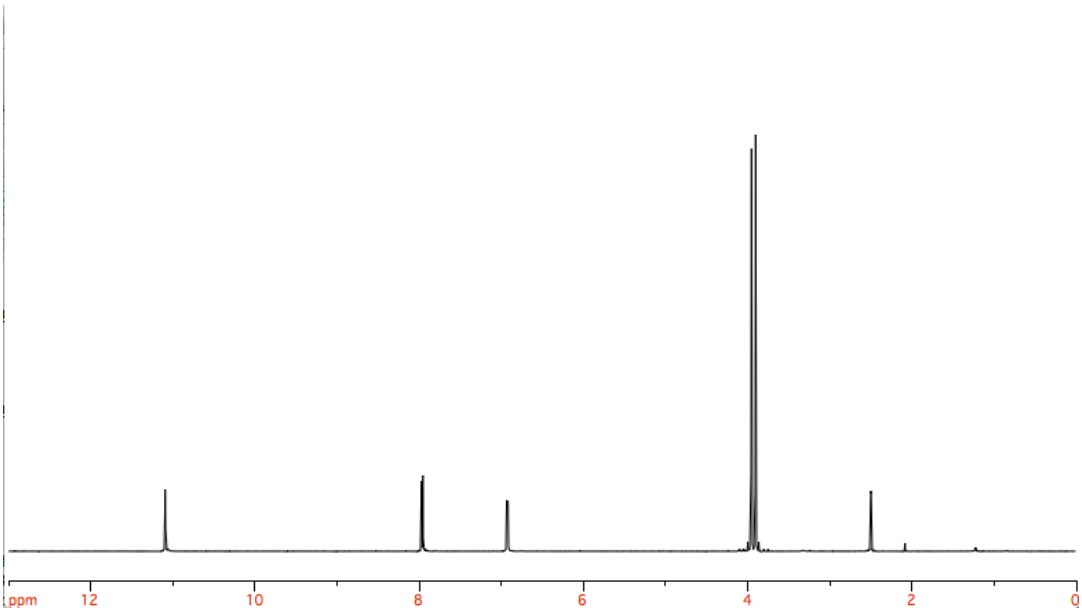
A mixture of methyl 2-hydroxy-4-methoxybenzoate (1.00 g, 5.49 mmol) and [^{15}N]-$Ca(NO_3)_2$ (1.80 g, 11.0 mmol) in 5 mL of glacial acetic acid was heated at 400 MW power in a domestic microwave for 30 sec. Five mL of ice-cold water was then added and the reaction mixture was incubated in an ice-water bath for 30 min. The solid was then filtered, washed with 20 mL of ice-cold water, and dried *in vacuo* to afford methyl 2-hydroxy-4-methoxy-3-[^{15}N]nitrobenzoate (147.7 mg, 0.6500 mmol, 11.8%) as a pale yellow solid.

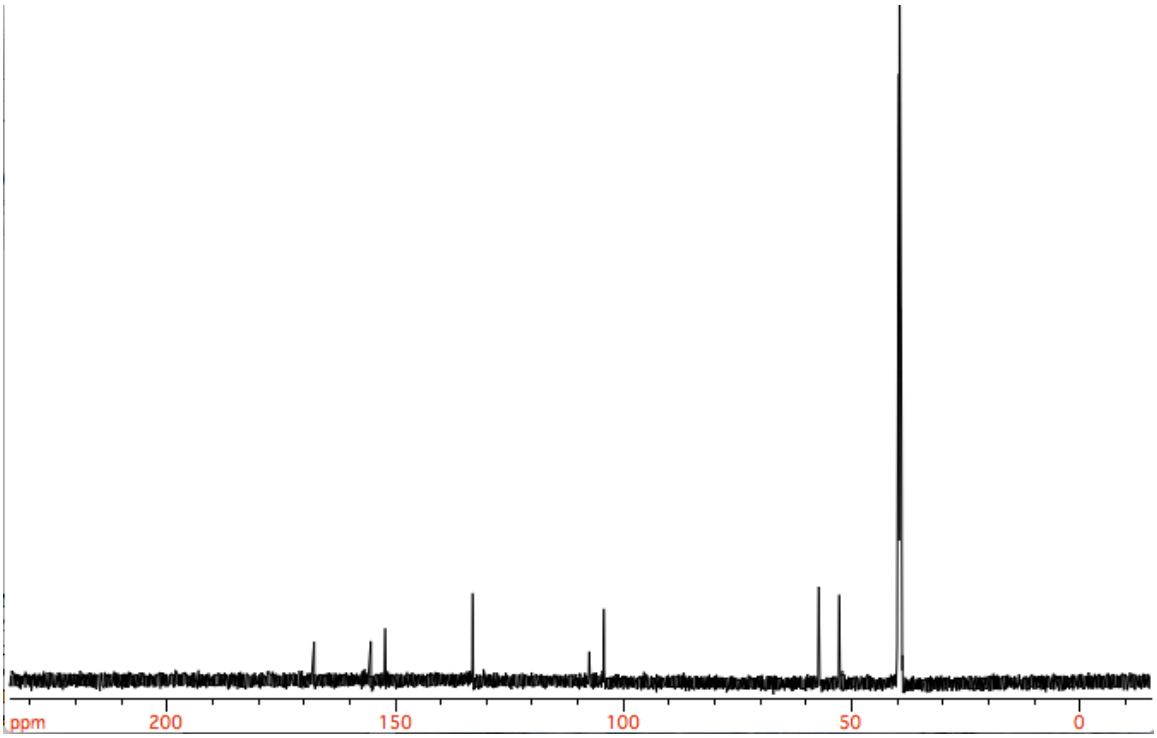
HRMS (ESI): calc'd for $C_9H_{10}{}^{15}NO_6^+$ $[M+H]^+$, 229.0473; found, 229.0469

^{1}H NMR (500 MHz, d_6-DMSO) δ: 11.09 (s, 1H, -O**H**), 7.96 (d, J = 9.09 Hz, 1H, aromatic 6-**H**), 6.92 (d, J = 9.14 Hz, 1H, aromatic 5-**H**), 3.96 (s, 3H), 3.91 (s, 3H)

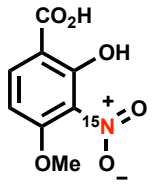
^{13}C NMR (125 MHz, d_6-DMSO) δ: 167.78 (**C**OOCH3), 155.37 (C4), 152.25 (C2), 133.07 (C6), 130.6 (d, J = 17.5 Hz, C3) 107.51 (C1), 104.30 (C5), 57.23 (OCH3), 52.75 (COO**C**H3)


CO2Me
OH
+
O
15N
OMe
O_


ppm
12
10
8
6
4
2
0


ppm
200
150
100
50
0

2-Hydroxy-4-methoxy-3-[^{15}N]nitrobenzoic acid



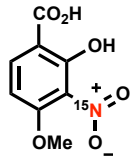
The synthesis of 2-hydroxy-4-methoxy-3-[^{15}N]nitrobenzoic acid was accomplished by following the procedure previously reported by Varley *et al* except for the use of ^{15}N-labeled substrate.[8]

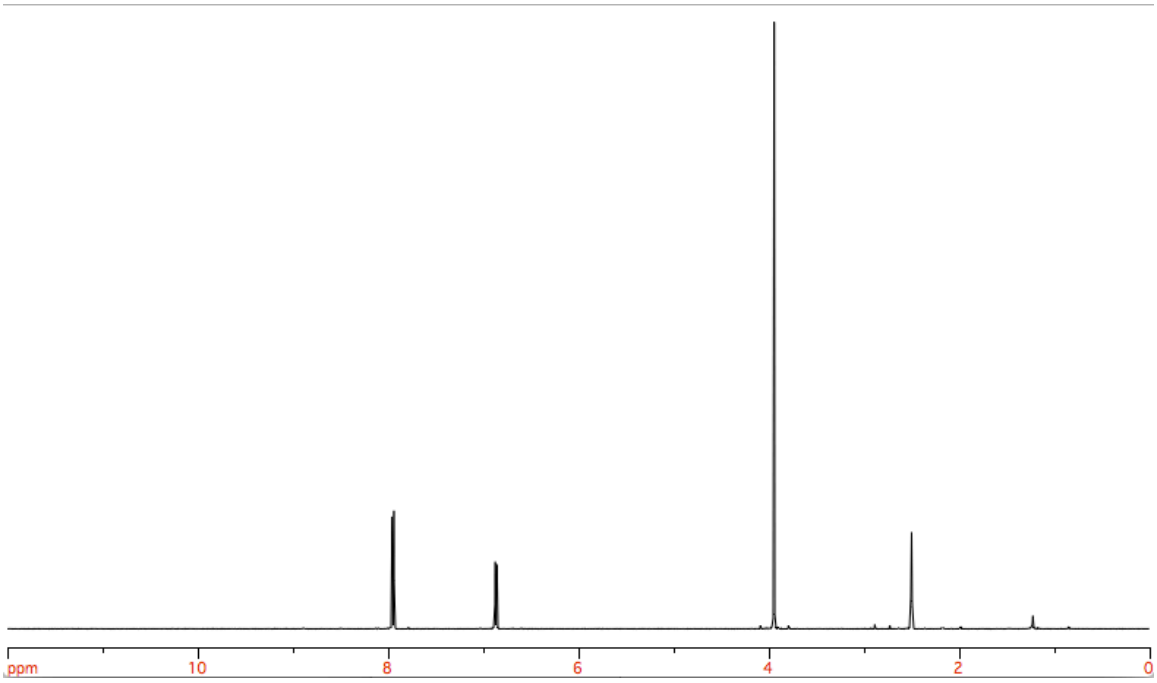
To a solution of methyl 2-hydroxy-4-methoxy-3-[^{15}N]nitrobenzoate (140 mg, 0.613 mmol) in THF (28 mL) and water (7 mL) was added solid LiOH (805 mg, 33.7 mmol). The resulting reaction mixture was heated under reflux with stirring overnight. After cooling to room temperature, the mixture was acidified to pH 2 with concentrated HCl and then extracted with dichloromethane (3 x 25 mL). The organic extracts were combined, dried over Na_2SO_4, and concentrated to yield 2-hydroxy-4-methoxy-3-[^{15}N]nitrobenzoic acid as a pale yellow solid which was used without further purification.

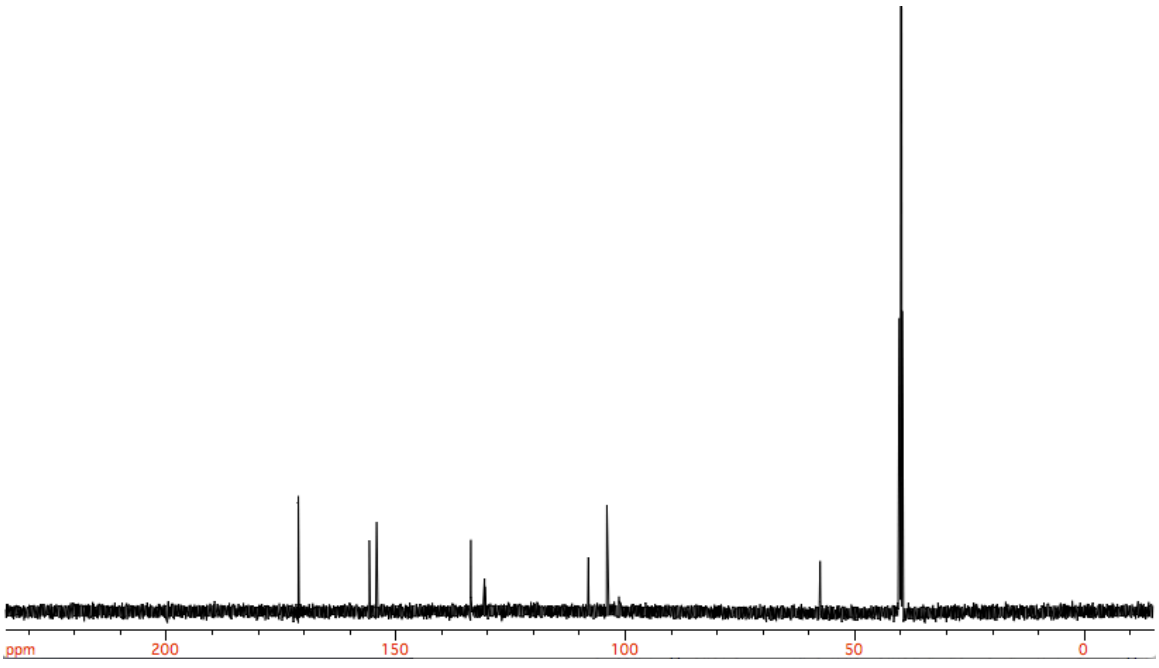
HRMS (ESI): calc'd for $C_8H_6{}^{15}NO_6^-$ $[M-H]^-$, 213.0171; found, 213.0175

^{1}H NMR (500 MHz, d_6-DMSO) δ: 7.95 (d, J = 9.04 Hz, 1H, aromatic 6-**H**), 6.87 (d, J = 9.08 Hz, 1H, aromatic 5-**H**), 3.94 (s, 3H, C**H$_3$**)

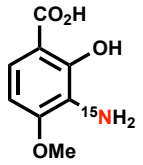
^{13}C NMR (125 MHz, d_6-DMSO) δ: 171.25 (COOH), 155.77 (C4), 154.19 (C2), 133.68 (d, J = 5 Hz, C6), 130.61 (d, J = 17.5 Hz, C3), 108.07 (C1), 104.03 (C5), 57.59 (d, J = 10.0 Hz, CH3)


CO2H
OH
15N
O
OMe
O


ppm
10
8
6
4
2
0


ppm
200
150
100
50
0

[^{15}N]-3-Amino-2-hydroxy-4-methoxybenzoic acid



The synthesis of [^{15}N]-3-amino-2-hydroxy-4-methoxybenzoic acid was accomplished by following the procedure previously reported by Varley *et al* except for the use of ^{15}N-labeled substrate.[8]

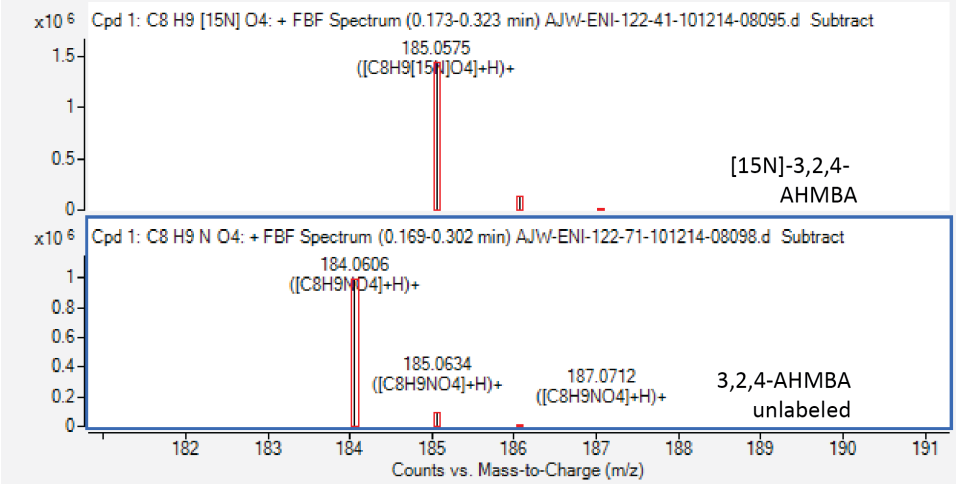
To a crude solution of 2-hydroxy-4-methoxy-3-[^{15}N]nitrobenzoic acid in EtOH (15 mL) was added Pd/C (70 mg, 10 weight %). The reaction mixture was vigorously stirred under an atmosphere of H_2 overnight. The mixture was then filtered through Celite and the Celite was rinsed with EtOH (100 mL) to elute the desired compound. The solution was concentrated *in vacuo* to yield [^{15}N]-3-amino-2-hydroxy-4-methoxybenzoic acid (113.8 mg, 100% yield over two steps) as a dark brown solid.

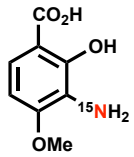
HRMS (ESI): calc'd for $C_8H_{10}{}^{15}NO_4^+$ $[M+H]^+$, 185.0575; found, 185.0575

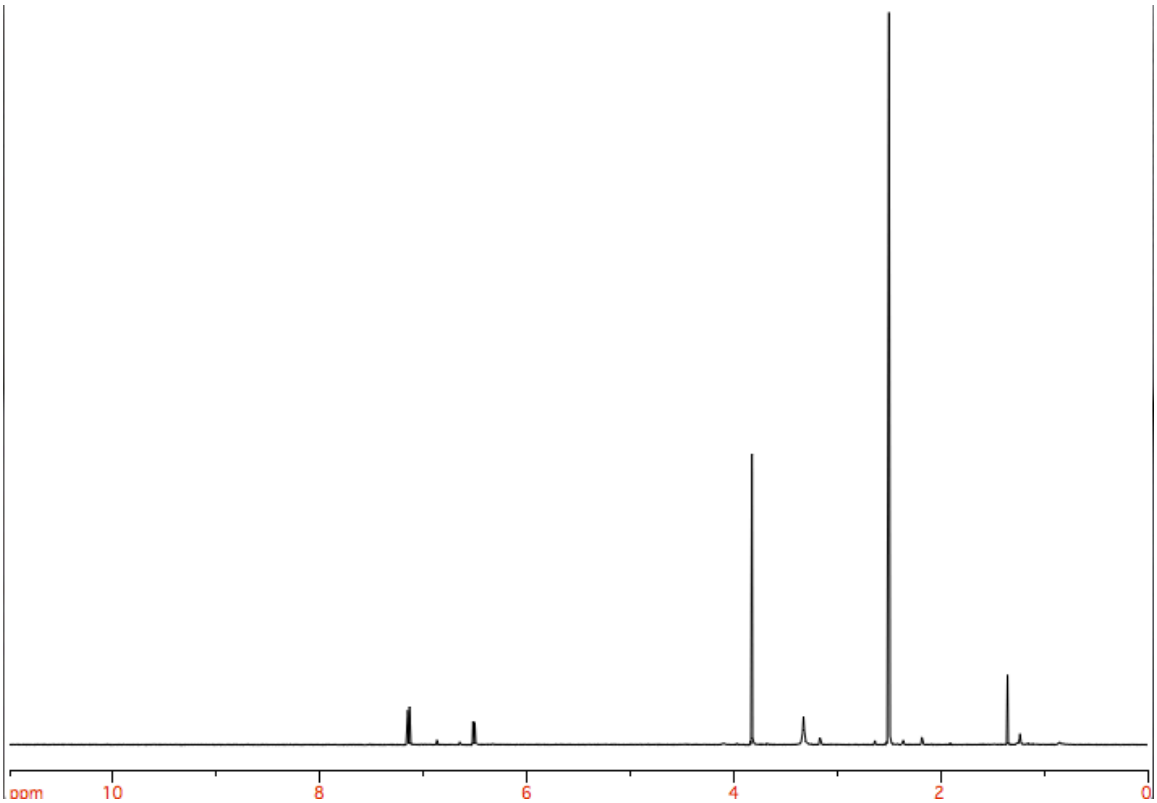
^{1}H NMR (500 MHz, d_6-DMSO) δ: 7.14 (d, J = 8.90 Hz, 1H, aromatic 6-**H**), 6.51 (d, J = 8.85 Hz, 1H, aromatic 5-**H**), 3.82 (s, 3H, C**H$_3$**)

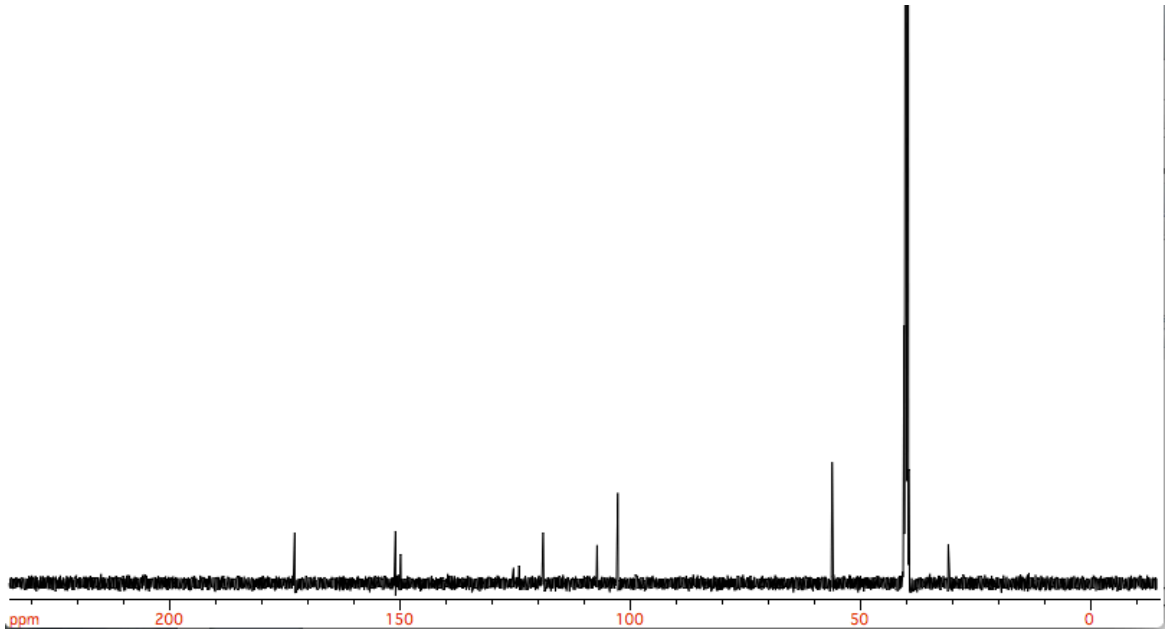
^{13}C NMR (125 MHz, d_6-DMSO) δ: 172.94 (COOH), 152.03 (C4), 149.91 (C2), 124.12 (J = 11.3 Hz, C3), 118.94 (C6), 107.21 (C1), 102.7 (C5), 56.10 (CH3)

The sample was 99% enriched in ^{15}N as determined by HRMS (ESI).

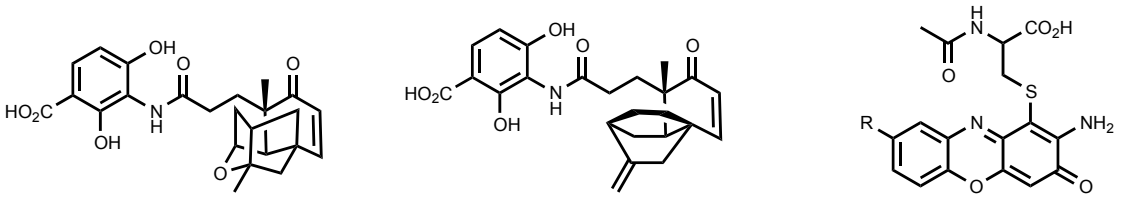








13. Selected 3,4-AHBA containing natural products



platensimycin

platencin

R = -CHO : grixazone A
R = -CO_2H : grixazone B

Figure S12. Structures of selected 3,4-AHBA-derived natural products.[13-18]

References

[1] M. E. Bergy, T. R. Pyke, US Patent 3350269, **1967**.

[2] J. N. McGuire, S. R. Wilson, K. L. Rinehart, *J. Antibiot*. **1995**, *48,* 516.

[3] Y-H. Zhang, J-Q. Yu, *J. Am. Chem. Soc*. **2009**, *131*, 14654.

[4] A. Noguchi, T. Kitamura, H. Onaka, S. Horinouchi, Y. Ohnishi, *Nat. Chem. Biol*. **2010**, *6*, 641

[5] J. M. Winter, A. L. Jansma, T. M. Handel, B. S. Moore, *Angew. Chem. Int. Ed.* **2009**, *48*, 767.

[6] J. Gao, K. Ju, X. Yu, J. E. Velásquez, S. Mukherjee, J. Lee, C. Zhao, B. S. Evans, J. R. Doroghazi, W. W. Metcalf, W. A. van der Donk, *Angew. Chem. Int. Ed.* **2013**, *53*, 1334.

[7] J. Hayashida, V. H. Rawal, *Angew. Chem. Int. Ed.* **2008**, *47*, 4373.

[8] L. S. Varley, C. J. Moody, *Synthesis*, **2008**, *22*, 3601.

[9] Y. Mao, W. Zhu, X. Kong, Z. Wang, H. Xie, J. Ding, N. K. Terret, J. Shen, *Bioorg. Med. Chem.* **2013**, *21*, 3090.

[10] H. Suzuki, Y. Ohnishi, Y. Furusho, S. Sakuda, S. Horinouchi, *J. Biol. Chem.* **2006**, *281*, 36944.

[11] A. K. Bose, S. N. Ganguly, M. S. Manhas, S. Rao, J. Speck, U. Pekelny, E. Pombo-Villars, *Tetrahedron Letters*. **2006**, *47*, 1885.

[12] D. Xiao, L. Zhu, Y. Hu, International Patent Application WO2013/97773 A1, **2013**.

[13] H. Jayasuriya, K. B. Herath, C. Zhang, D. L. Zink, A. Basilio, O. Genilloud, M. T. Diez, F. Vicente, I. Gonzalez, O. Salazar, F. Pelaez, R. Cummings, S. Ha, J. Wang, S. B. Singh, *Angew. Chem. Int. Ed.* **2007**, *46*, 4684.

[14] S. B. Singh, H. Jayasuriya, J. G. Ondeyka, K. B. Herath, C. Zhang, D. L. Zink, N. N. Tsou, R. G. Ball, A. Basillo, O. Genilloud, M. T. Diez, F. Vicente, F. Pelaez, K. Young, J. Wang, *J. Am. Chem. Soc.* **2006**, *128*, 11916.

[15] S. J. Gould, C. R. Melville, M. C. Cone, *J. Am. Chem. Soc.* **1996**, *118*, 9228.

[16] A. M. Hassan, M. C. Cone, C. R. Melville, S. J. Gould, *Bioorg. Med. Chem. Lett.* **1995**, *5*, 191.

[17] S. Imai, A. Shimazu, K. Furihata, K. Furihata, Y. Hayakawa, H. Seto, *J. Antiobiot.* **1990**, *12*, 1606.

[18] Y. Ohnishi, Y. Furusho, T. Higashi, H. Chun, K. Furihata, S. Sakuda, S. Horinouchi, *J. Antibiot*. **2004**, *57*, 218.